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Regulation of PCNA Function by Tyrosine Phosphorylation in Prostate Cancer

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14. ABSTRACT
The proliferating cell nuclear antigen (PCNA) has been widely used as a molecular marker for tumor progression and patient prognosis in a variety of human cancers. The PCNA protein has two forms: a non-functional chromatin-unbound form, and a functional chromatin-associated form. The latter form is associated with cancer and tumor progression. Currently, there are no assays which can distinguish these two, distinct forms of the PCNA protein. As a result of an inability to discriminate between the functional and non-functional types, relating PCNA levels in tumor tissues with patient prognosis has been difficult. The Principal Investigator (PI) recently discovered that the functional, chromatin-bound form of PCNA is phosphorylated at tyrosine residue Y211, and that the nonfunctional form is not phosphorylated at this residue. This distinguishing characteristic could provide a means for reliably differentiating between the two protein forms and allowing healthcare providers to utilize PCNA levels to make a more accurate assessment of tumor progression and patient prognosis. Additionally, the PI found that specifically inhibiting Y211 phosphorylation resulted in the degradation of the functional form of PCNA and suppressed its DNA synthesis and repair functions. This promising result suggests that blocking Y211 phosphorylation of PCNA could provide a means for inhibiting tumor growth in cancers in the future. The key objectives of this research are: (1) Determine if blocking phospho-Y211 PCNA can lead to the suppression of prostate cancer cell proliferation and enhancement of chemosensitivity in prostate cancer cells; (2) Examine whether phospho-Y211 PCNA is a good prognosis marker and whether its level in tissues is associated with key pathological characteristics of prostate cancer.

15. SUBJECT TERMS
PCNA, Tyrosine Phosphorylation, Prostate Cancer

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Table of Contents

Introduction

The underlying hypothesis of this research is that blocking Y211 phosphorylation of PCNA can provide a targeted approach to inhibit tumor growth and sensitize tumor response to combination chemotherapy in prostate cancer. In addition, the chromatin-bound form of PCNA, which can be specifically recognized by the anti-phospho-Y211 PCNA antibody, is the authentic form for cell proliferation and should be a novel prostate tumor marker associated with poor prognosis.

Body

Task 1: Develop a novel therapeutic approach by targeting Y211 phosphorylation of PCNA in prostate cancer cells.

a) *Inhibit Y211 phosphorylation of PCNA in the prostate cell lines and examine the effects on cell proliferation and in modulating response to combination chemotherapy.*

As shown in the data presented below, we have develop a Y211F peptide of PCNA in this study. This peptide has the following activities in prostate cancer cells:
(1) inhibition of Y211 phosphorylation of PCNA. (Figure 1)

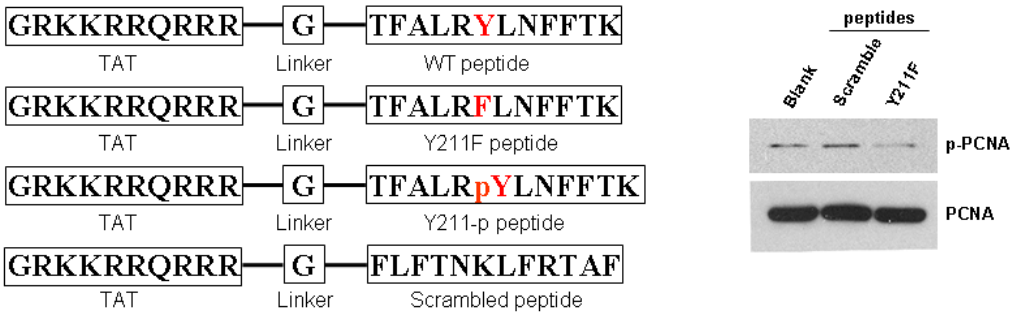


Figure 1. *Left panel, the schematic demonstration of the peptides used in this study. The Y211 residue and the substitutions are indicated in red. Right panel, PC-3 cells were treated with 15 μM of the indicated peptides or mock treated. The endogenous phospho-Y211 PCNA was immunoprecipitated from 1 mg of cell lysate with 2 μg of the anti-phospho-Y211 antibody. The pulled down phospho-Y211 PCNA was separated by electrophoresis and blotted with anti-PCNA antibody.*

(2) suppression of cell growth. (Figures 2 and 3)

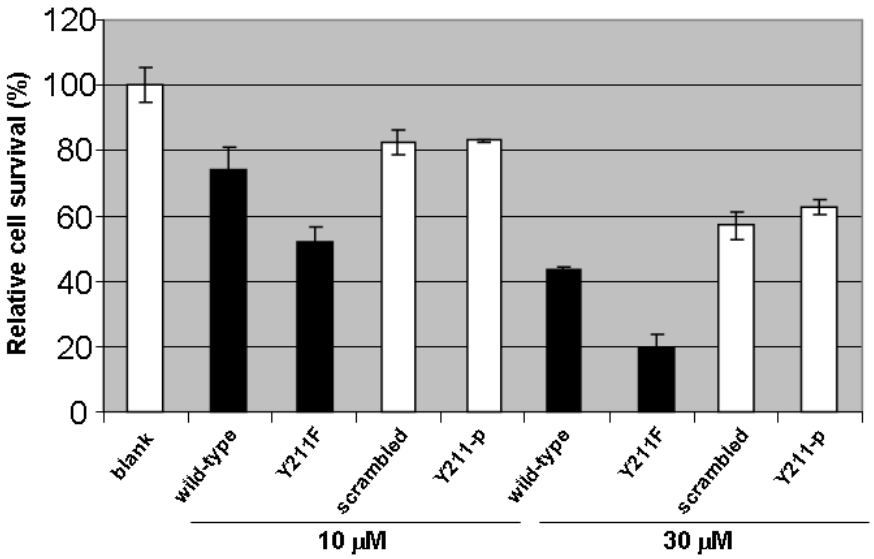


Figure 2. *PC-3 cells were seeded in 96-well plates (3000 cells per well). The cells were then treated with the indicated peptide and dose for 72 hours, followed by MTT assay to assess the number of viable cells with and without the peptide treatment. It is noted that the cells were sensitive specifically to the wild-type and Y211F peptides but not the scrambled and the Y211-p peptides.*

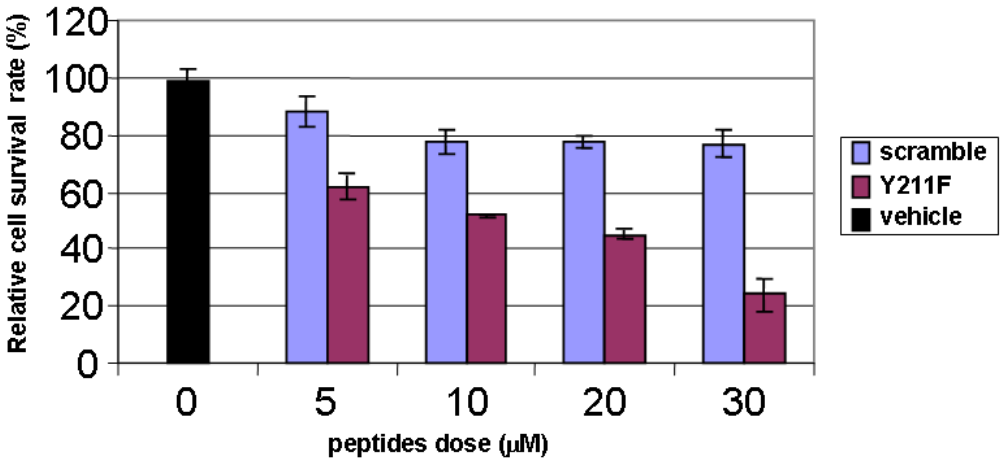


Figure 3. *PC-3 cells were specifically sensitive to the growth inhibitory effect of the Y211F peptide. Cells were plated and treated as described in Figure 2 except that the cells were treated for 48 hr. This data demonstrated that the Y211F peptide is a potent cell growth inhibitor.*

(3) inhibition of cell proliferation (DNA replication activity). (Figure 4)

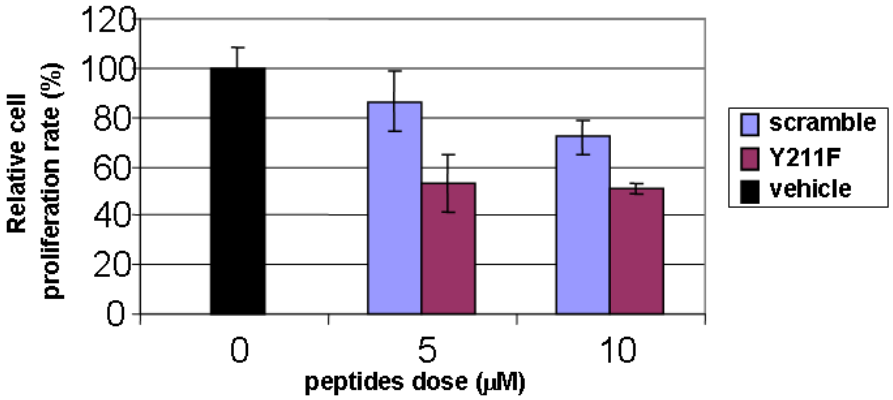


Figure 4. *The Y211F peptide inhibits the proliferation of PC-3 cells. Cells were treated with the peptides or mock treated (vehicle) and then subject to the assay of BrdU incorporation using a kit (Roche; Indianapolis, IN). For this assay, cells were incubated with BrdU (10 μM) for 6 hr. The cells were then fixed with ethanol and the genomic DNA was denatured. The treated cells were then incubated with peroxidase-conjugated anti-BrdU antibody and the labeling can then be determined by colorimetric measurement with a ELISA plate reader at 370 nm.*

(4) sensitization of the prostate cancer cells to genotoxic stresses. (Figure 5)

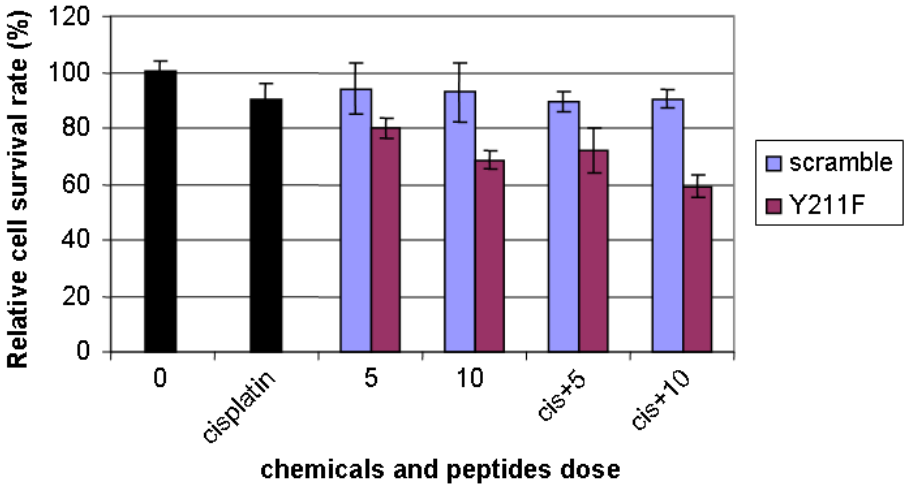


***Figure 5**. Y211F peptide sensitize PC-3 cells to cisplatin treatment. PC-3 cells were plated in 96-well as described in Figure 2. The cells were then mock treated, or treated by cisplatin alone (10 μM), the peptide alone (5 or 10 μM), or in combination with 10 μM of cisplatin at the indicated concentration for 72 hr. While treatment by the scrambled peptide did not affect the sensitivity of cells to cisplatin, co-treatment by the Y211F peptide significantly reduce the viability of PC-3 cells in response to cisplatin.*

(5) The inhibition of Y211 phosphorylation and the growth inhibition can be extended to other two prostate cancer cell lines. DU145 and LNCaP. (Figure 6 and 7)

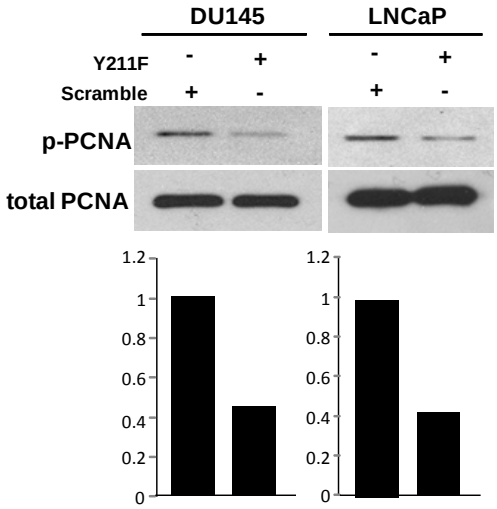
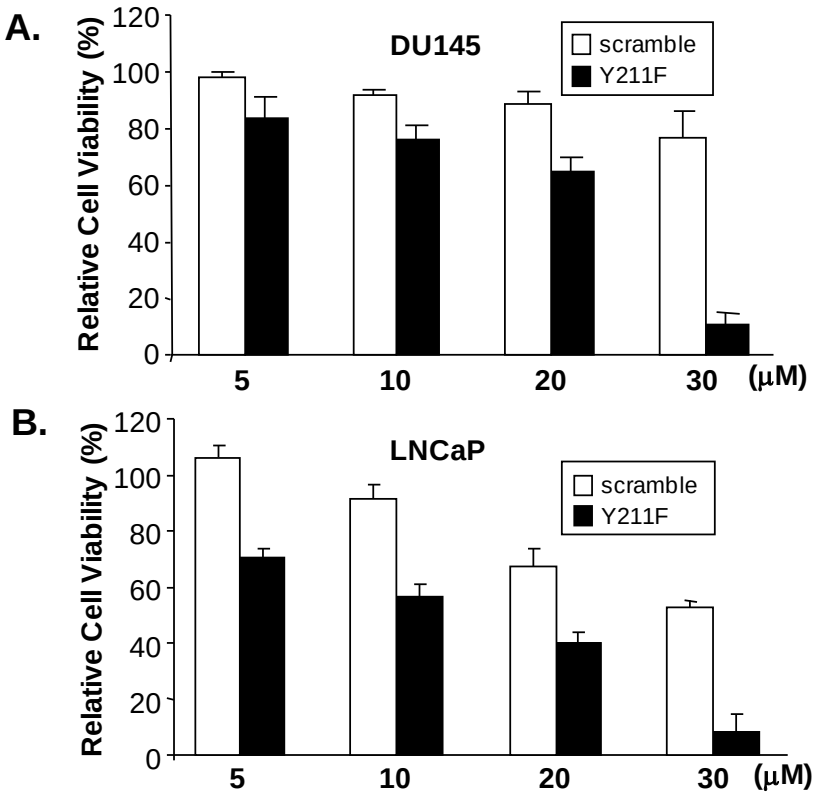


***Figure 6.** The Y211F peptide inhibited Y211 phosphorylation of PCNA in prostate cancer cells. The prostate cancer cell lines LNCaP and DU145 were treated with 15 μM of the Y211F peptide, or the scramble peptide or vehicle alone as controls, for 12 h. Cells were then lysed and phospho-Y211 PCNA was immunoprecipitated by using the anti-phospho-Y211 antibody and was examined by western blotting with the anti-PCNA antibody. The input amount of PCNA in the lysates was also determined. Refer to Figure 1 for the effect on Y211 phosphorylation in PC-3 cells.*

***Figure 7.** Inhibition of Y211 phosphorylation suppressed prostate cancer growth. The prostate cancer cell lines DU145 **(A)** and LNCaP **(B)** were plated in 96-well plates and then incubated for 48 h with the indicated peptides at different concentrations. The numbers of viable cells exposed to each treatment was then determined and expressed relative to vehicle-treated cells. For the effect in PC3 cells, refer to Figure 3.*



(6) The importance of down-regulation of Y211 phosphorylation in the growth suppression activity of the Y211F peptide was demonstrated by the observation that a phosphomimetic PCNA mutant (Y211E) conferred resistance to the peptide. (Figure 8)

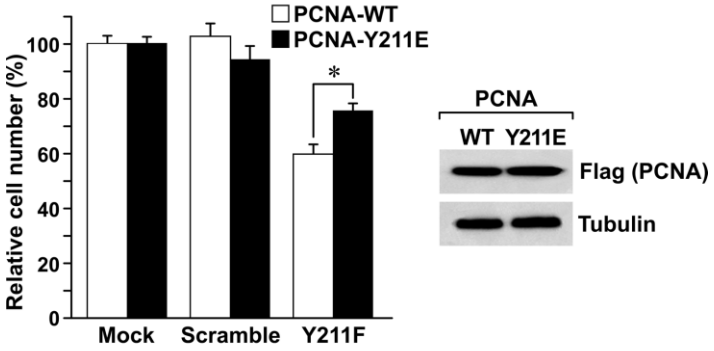


Figure 8. *Phosphomimetic PCNA was resistant to growth inhibition mediated by the Y211F peptide. PC3 cells were transiently transfected with FLAG-tagged wild-type (black bars) or Y211E (white bars) PCNA, and then treated with the Y211F peptide or the scramble control peptide at 5 μM, or vehicle alone, for 10 h. Left panel, cells expressing the Y211E phosphomimetic mutant PCNA were less sensitive to the growth inhibition effect of the Y211F peptide than cells expressing the wild-type PCNA as determined by the cell viability assay. *, P < 0.05. Right panel, expression levels of the transfected PCNA were determined by western blotting analysis*.

(7) arrest of cell cycle progression of prostate cancer cells by the Y211F peptide. (Figure 9)

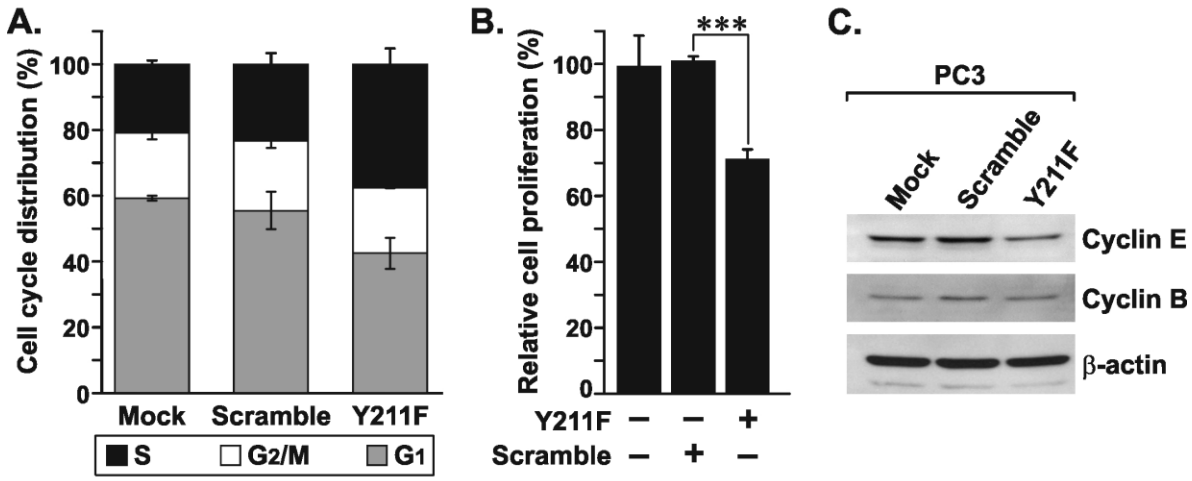


Figure 9. *Y211F peptide treatment induced S-phase arrest and reduced cell proliferation.* ***A.*** *PC3 cells mock treated with vehicle alone, or treated with the scramble or Y211F peptide (15 μM) were subjected to flow cytometry analysis. The percentage of cells in the G1, S, and G2/M phases were plotted.* ***B.*** *DNA synthesis activity in the treated cells was determined by a colorimetric BrdU-incorporation analysis. For each data point the amount of cells was normalized by a side-by-side assay for viable cells. ***, P < 0.001.* ***C.*** *Expression of cyclin E and cyclin D was determined by western analysis. β-actin was determined as the internal control.*

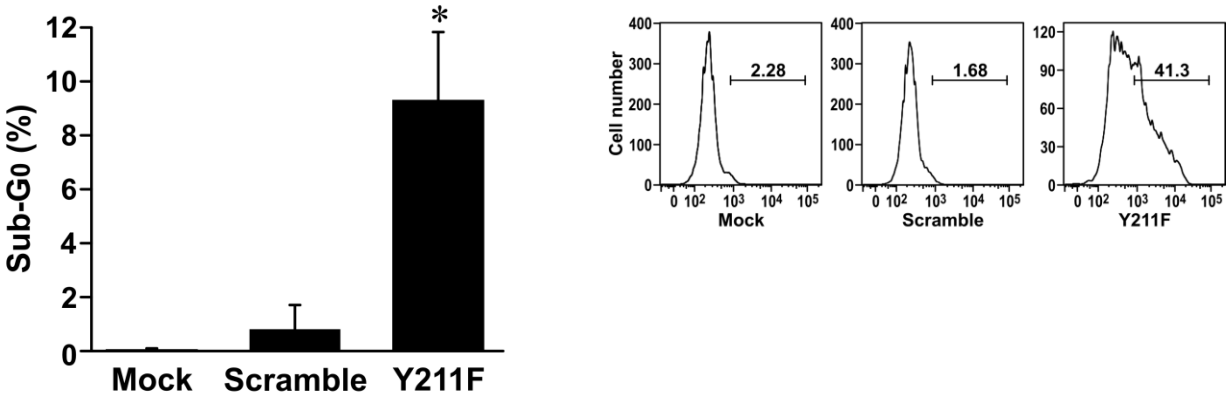
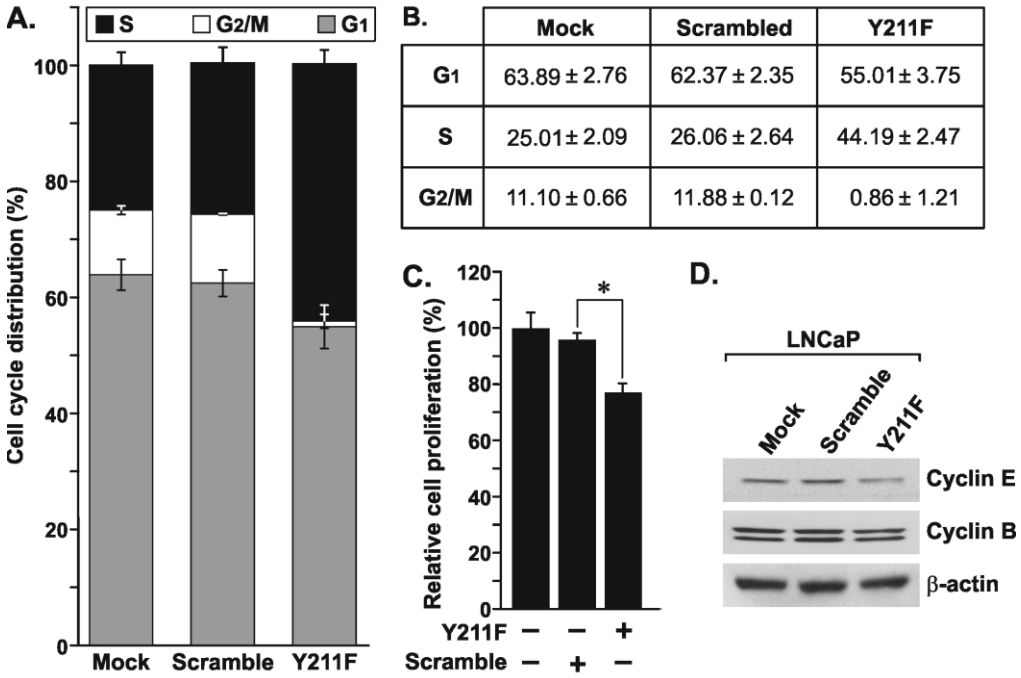


Figure 9. *Y211 peptide treatment caused cell death in PC3 cells.* ***A.*** *The graph shows the proportion of PC3 cells in the sub-G0 phase, as assessed by flow cytometry, after the indicated treatment. *, P < 0.05.* ***B.*** *To confirm cell death, cells were labeled with annexin V (BD; San Jose, CA) after the indicated treatment, and the positive-staining cells were measured by flow cytometry.*

Our next question was to determine whether the effect of Y211F peptide on cell cycle progression is cell line-specific or can be applied to other prostate cancer cells. Here we show that treating LNCaP cells with the Y211F peptide resulted in similar cell cycle arrest at S phase, inhibition of cell proliferation, and down-regulation of cyclin E (Figure 11).



	Mock	Scrambled	Y211F
G1	63.89 ± 2.76	62.37 ± 2.35	55.01 ± 3.75
S	25.01 ± 2.09	26.06 ± 2.64	44.19 ± 2.47
G2/M	11.10 ± 0.66	11.88 ± 0.12	0.86 ± 1.21

Figure 11. *Y211F peptide treatment induced S-phase arrest and reduced cell proliferation in LNCaP cells.* ***A.*** *Cells treated with the scramble peptide (15 μM), the Y211F peptide (15 μM), or vehicle alone were subject to flow cytometry analysis as described in Figure 4.* ***B.*** *Cell-cycle distributions of cells exposed to the indicated treatments. The results were derived from three independent experiments.* ***C.*** *The DNA-synthesis activity of the treated cells was determined by BrdU incorporation assay as described in Figure 4 and the Materials and Methods section. *, P < 0.05.* ***D.*** *Expression of cyclin E and cyclin D in the treated cells was determined by western analysis. Tubulin is shown as the internal control.*

Furthermore, the Y211F peptide caused significant level of cell death, as demonstrated by the proportion of sub-G0 cells and annexin V-positive cells after flow cytometry (Figure 12).

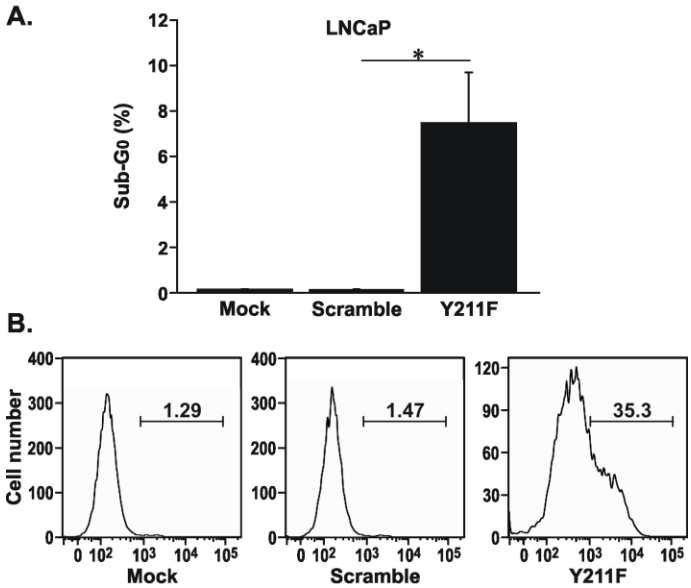
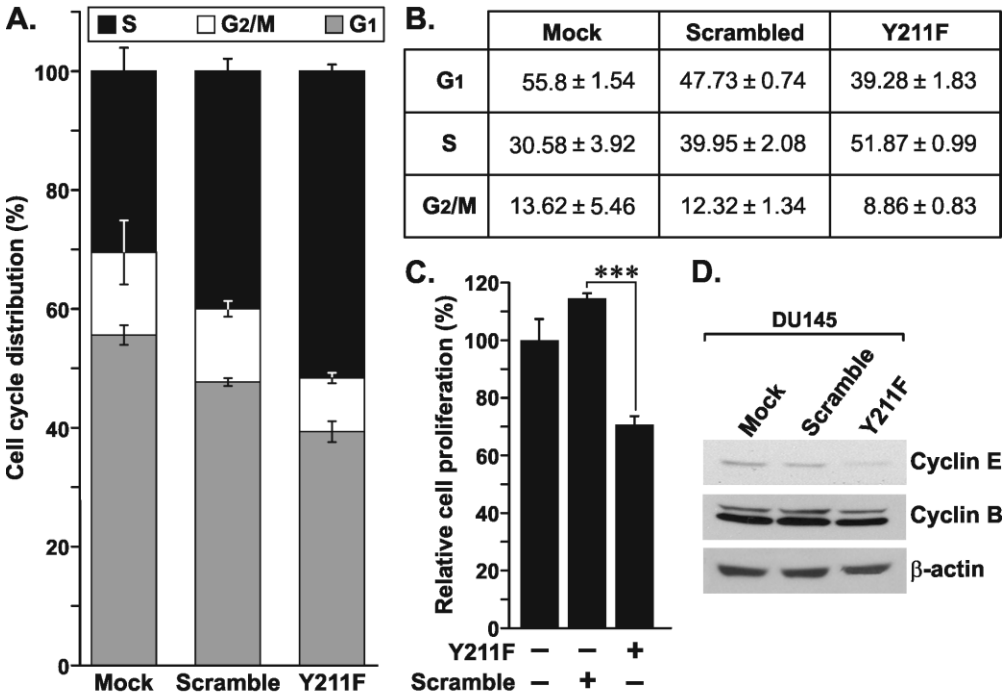


Figure 12. *Y211 peptide treatment caused cell death in LNCaP cells.* ***A.*** *The graph shows the proportion of LNCaP cells in the sub-G0 phase, as assessed by flow cytometry, after the indicated treatment. *, P < 0.05.* ***B.*** *To confirm cell death, cells were labeled with annexin V (BD; San Jose, CA) after the indicated treatment, and the positive-staining cells were measured by flow cytometry.*

Similar results were observed for another prostate cancer cell line DU145 (Figures 13 and 14)



	Mock	Scrambled	Y211F
G1	55.8 ± 1.54	47.73 ± 0.74	39.28 ± 1.83
S	30.58 ± 3.92	39.95 ± 2.08	51.87 ± 0.99
G2/M	13.62 ± 5.46	12.32 ± 1.34	8.86 ± 0.83

Figure 13. *Y211F peptide treatment induced S-phase arrest and reduced cell proliferation in DU145 cells, as described in Figure 4 and the Materials and Methods section. ***, P < 0.001.*

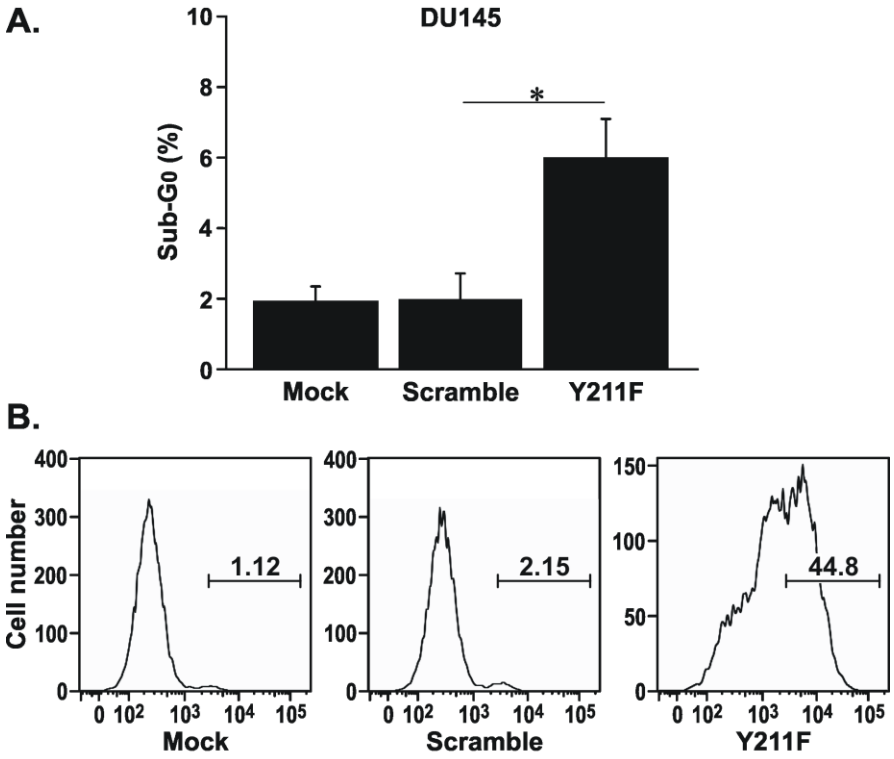


Figure 14. *Y211 peptide treatment caused cell death in DU145 cells, as described in Figure 4 and the Materials and Methods section. *, P < 0.05.*

b) *Treat PC3 cells with the peptides and test the effect on tumorigenesis*

We have successfully conducted this animal experiments. To do this, we tested the growth-suppressing activity of the peptide in tumor xenografts derived from PC3 cells implanted subcutaneously into nude mice (**Fig. 15**). Treatment by the Y211F peptide, but not by the mock treatment with the vehicle alone or by the control scramble peptide, significantly suppressed tumor growth, as measured by tumor volume (**Fig. 15A**), as well as by the actual weight of isolated tumors (**Fig. 15, B and C**). Immunohistochemical staining using the anti-phospho-Y211 antibody confirmed the down-regulation of Y211 phosphorylation of PCNA in the tumors injected with the Y211F peptide, in contrast to the tumor tissues injected with vehicle alone or the scramble peptide (**Fig. 15D**). These results corroborate with the in vitro data and demonstrate the importance of Y211 phosphorylation in prostate cancer cell growth as well as its potential as a target for cancer therapy.

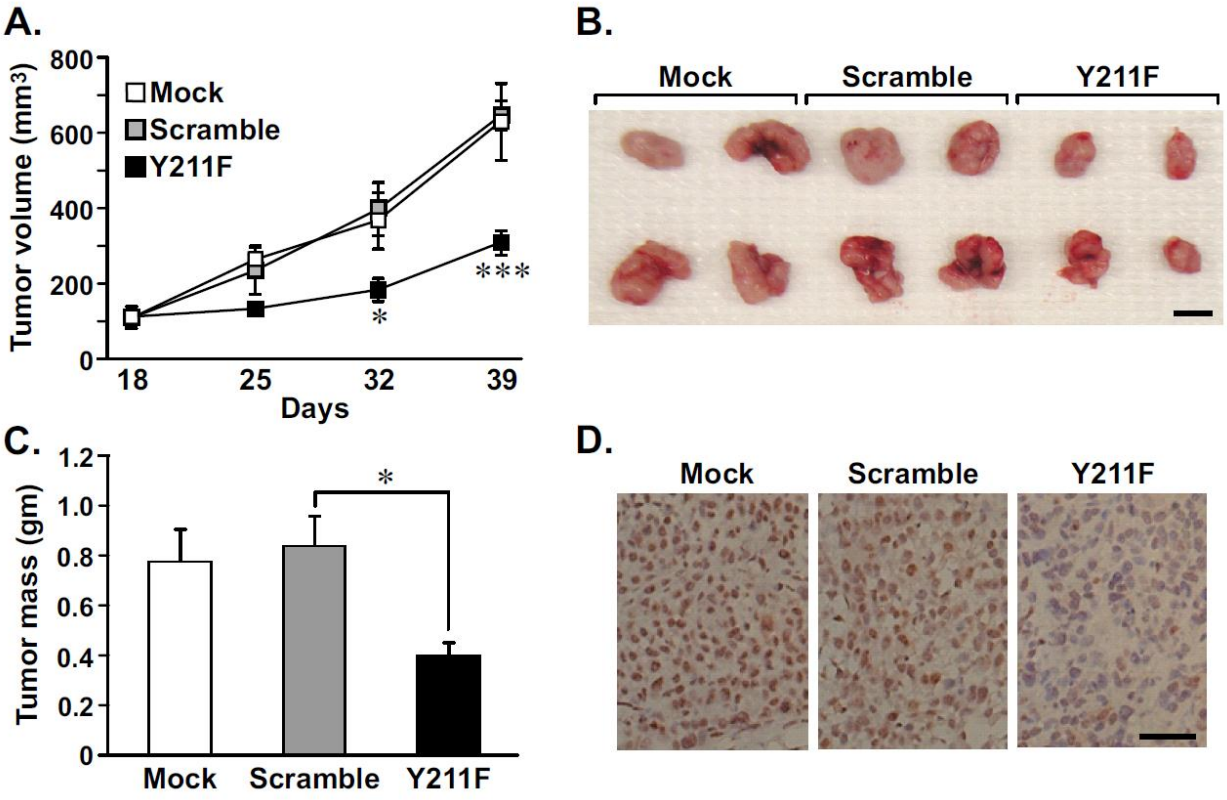


Figure 15. *Y211 peptide treatment suppressed tumor growth and Y211 phosphorylation in a xenograft model of prostate cancer tumors.* ***A.*** *2.5x10^6 PC3 cells were injected subcutaneously into the flanks of nude mice. When the tumors were palpable, mice were grouped randomly into three groups with four mice in each group. The mice were then treated with control vehicle (Mock), the control peptide (Scramble), or the Y211F peptide (Y211F), by intratumoral injection. Tumor growth was monitored weekly by measuring tumor size. *, P < 0.05; ***, P < 0.001.* ***B.*** *Tumors were isolated from the mice at the end of treatment. Photographs of representative tumors are shown. Bar, 1 cm.* ***C.*** *The weight of each of the harvested tumors was measured and plotted. Treatment with the Y211F peptide significantly decreased the size of the tumors. *, P < 0.05.* ***D.*** *Tumor tissues were fixed by formaldehyde and embedded in paraffin. Tissues sections were then stained with the anti-phospho-Y211 PCNA antibody. Bar, 50 µm.*

Task 2: Determine the importance of Y211 phosphorylation of PCNA as a prognosis marker of tumor progression in prostate cancer.

We have established in a pilot experiment that the anti-phospho-Y211 antibody is excellent for immunohistochemical staining studies. (Figure 16)

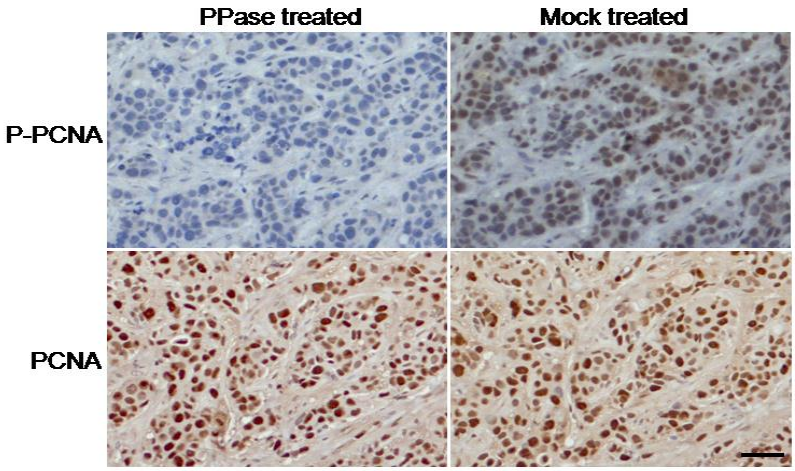


Figure 16. *In vivo staining for Y211 phosphorylation of PCNA. The micrographs show sequential sections of formalin-fixed and paraffin-embedded xenograft tumor tissue derived from the breast cancer cell line BT474. Sections were mock treated (with BSA and phosphatase enzyme buffer) or treated with lambda phosphatase and stained with the anti-phospho-Y211 antibody by immunohistochemistry (DAKO). Bar, 50 μm. Note that although phospho-Y211 PCNA staining is diminished after phosphatase treatment, the staining of total PCNA is not affected.*

We have successfully conducted the IHC staining in a cohort of prostate cancer cells. To assess the extent of PCNA Y211 phosphorylation in prostate cancer, a tissue microarray (TMA), consisting of tumor tissue derived from human prostate cancer patients, was screened by immunohistochemical (IHC) staining using the anti-phospho-Y211 antibody. In this array,18 of 38 (47.4%) tumors scored high for Y211 phosphorylation while 20 of 38 (52.6%) scored with negative to low for Y211 phosphorylation (**Fig. 17 and Table 1**). Although the small sample size prohibited the evaluation of whether there was a statistically significant correlation between phospho-Y211 PCNA staining and pathological parameters, we did note that all the stage IV patients (n=3) scored high for Y211 phosphorylation. Importantly, this study also demonstrated that Y211 phosphorylation of PCNA is a frequent event in prostate cancer. Interestingly, positive phospho-Y211 staining was observed in the six out of seven matched adjacent normal tissues included in the TMA while remains low or undetectable in normal tissue from healthy individuals (data not shown). In total, our IHC study suggests that phosphorylation of PCNA at Y211 is found in about half of human prostate cancers.

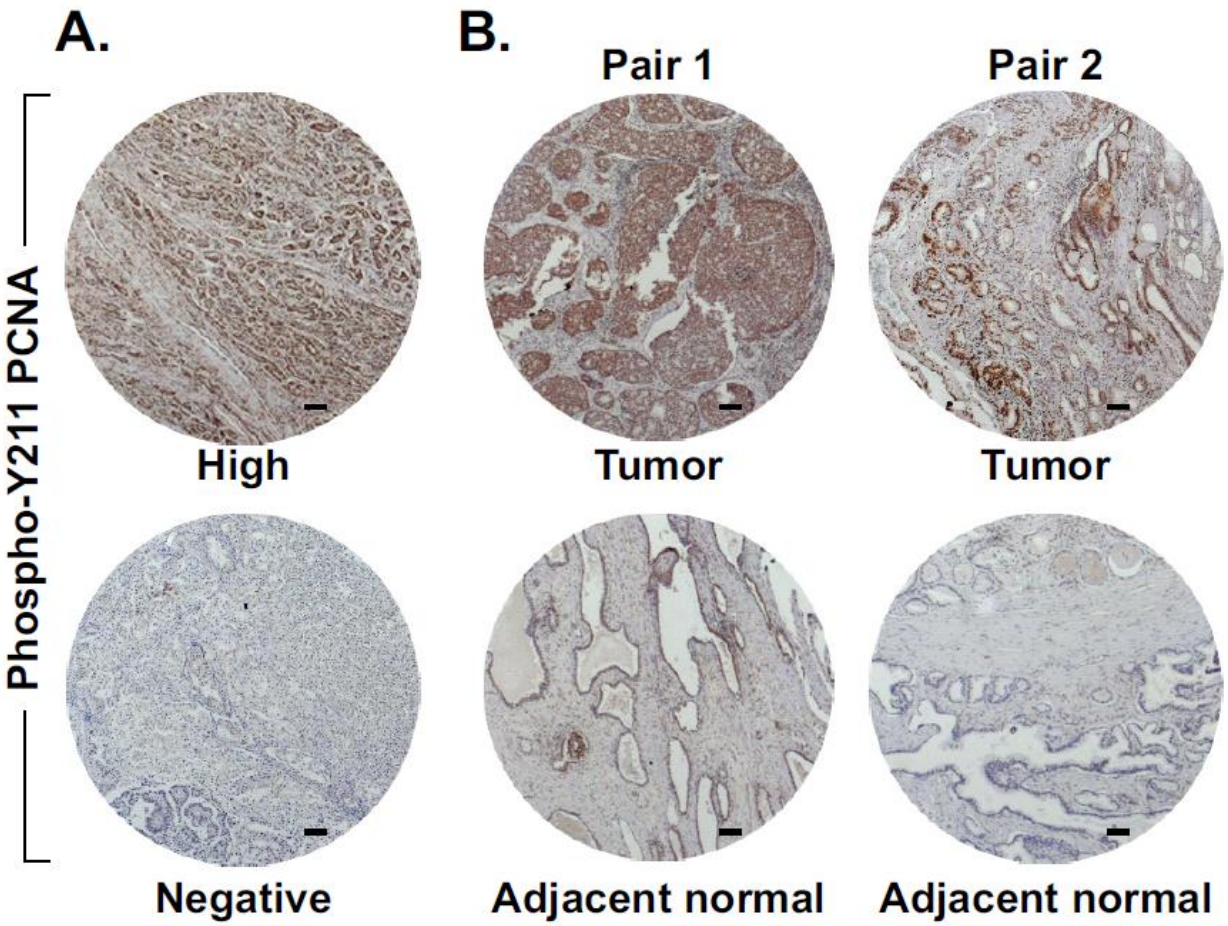


Figure 17. *Phosphorylation of PCNA at Y211 is frequently observed in prostate cancer. Human prostate cancer tissues were stained with an anti-phospho-Y211 PCNA antibody. Representative stained specimens are depicted. Bar, 0.5 mm.*

Table 1. Phospho-Y211 PCNA expression in human prostate specimens

PY211 Expression	% Samples	Mean Intensity	Intensity Range
High	47.4% (18/38)	202.5	120-285
Undetectable to Low	52.6% (20/38)	40.75	0-80

The percent of human prostate specimens that stained positive for phospho-Y211 expression and the mean intensity of phospho-Y211 staining are shown. The number of phospho-Y211–expressing samples over the total number of samples examined is shown in parentheses.

Key Research Accomplishments

- We have established that the cancer targeting activity of the Y211F peptide can be applied to other prostate cancer cells.
- The Y211F peptide causes cell cycle arrest, suppresses proliferation, and induces apoptosis.
- We have established that the Y211F peptide inhibit tumor growth of prostate cancer xenografts.
- We showed that Y211 phosphorylation is a frequent event in prostate cancer, but not in the normal tissues.

Reportable Outcomes

The study has resulted in a publication in prostate cancer from the PI's laboratory:

Zhao, H., Lo, Y.-H., Ma, L., Waltz, S.E., Gray, J.K., Hung, M.-C., Wang, S.-C. Targeting tyrosine phosphorylation of PCNA inhibits prostate cancer growth. *Molecular Cancer Therapeutics* 10:29-36, 2011.

The principle of inhibition of Y211 phosphorylation of PCNA and the use of the targeting peptide has also been applied to breast cancer:

Zhao, H., Lo, Y.-H., Ho, P.-H., Bedford, M.T., Hung, M.-C., and Wang, S.-C. Interaction of proliferation cell nuclear antigen (PCNA) with the non-receptor tyrosine kinase c-Abl in cell proliferation and response to DNA damages in breast cancer cells. *PLoS ONE*, 7(1): e29416. doi:10.1371/journal.pone.0029416, 2012. PMC3251568.

Both manuscripts are attached as appendixes.

Conclusion

We have demonstrated that the Y211F peptide is indeed a potent anti-prostate cancer agent. These results are important in that (1) they form the basis to develop efficient targeted therapeutic approaches for prostate cancer. Such approach may have potential to target the advanced prostate refractory to hormone therapies, which will be our next step to contribute to eliminating death of prostate cancer; and (2) they show that the phosphorylation of PCNA at Y211 may be a useful tumor marker for prostate cancer. Further directions include the investigation of the role of Y211 phosphorylation in hormone-refractory disease and develop systemic approach to deliver the therapeutic peptide.

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Molecular Cancer Therapeutics

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Molecular Cancer Therapeutics

Targeting Tyrosine Phosphorylation of PCNA Inhibits Prostate Cancer Growth

Huajun Zhao[1], Yuan-Hung Lo[1], Li Ma[1], Susan E. Waltz[1,2], Jerilyn K. Gray[1], Mien-Chie Hung[4,5,6], and Shao-Chun Wang[1]

Abstract

The proliferation cell nuclear antigen (PCNA) is a critical protein required for DNA replication in proliferating cells including cancer cells. However, direct inhibition of PCNA in cancer cells has been difficult due to the lack of targetable sites. We previously reported that phosphorylation of tyrosine 211 (Y211) on PCNA is important for the proliferative function of PCNA when this protein is associated with the chromatin in cancer cells. Here, we show that the Y211 phosphorylation of PCNA is a frequent event in advanced prostate cancer. To explore the potential of this signaling event in inhibition of cancer cell growth, we used a synthetic peptide, the Y211F peptide, which when present inhibits phosphorylation of Y211 on endogenous PCNA. Treatment with this peptide, but not a scrambled control peptide, resulted in S-phase arrest, inhibition of DNA synthesis, and enhanced cell death in a panel of human prostate cancer cell lines. In addition, treatment with the Y211F peptide led to decreased tumor growth in PC3-derived xenograft tumors *in vivo* in nude mice. Our study shows for the first time that PCNA phosphorylation at Y211 is a frequent and biologically important signaling event in prostate cancer. This study also shows a proof of concept that Y211 phosphorylation of PCNA may be used as a therapeutic target in prostate cancer cells, including cells of advanced cancers that are refractory to standard hormonal therapies. *Mol Cancer Ther; 10(1); 29–36. ©2011 AACR.*

Introduction

Prostate cancer is the most frequent cancer occurring in men in the United States and is the second leading cause of cancer deaths in men. It is estimated that in 2009, 192,280 new cases of prostate were diagnosed, and that 27,360 of these patients would succumb to this disease (American Cancer Society, Cancer Facts and Figures, 2009). Progression of prostate cancer follows a relentless pattern. During early-stage growth, cancer cells depend on androgen and, therefore, are sensitive to antiandrogen therapy. However, as the disease progresses, the tumor becomes resistant to androgen depletion and resumes active cell proliferation in the face of androgen deprivation. Currently, there is no cure for androgen-independent prostate cancer. In addition, a substantial proportion of patients with primary lesions localized to the prostate gland when first diagnosed can develop incurable disseminated disease after local therapy (1). Thus, there is a substantial need for new therapies that may target prostate cancer and the progression of this disease.

Proliferating cell nuclear antigen (PCNA) is the molecular coordinator for DNA replication and for maintaining genome integrity (2–10). PCNA forms a homotrimeric sliding clamp that encircles the chromatin and acts as a molecular platform to recruit proteins involved in DNA synthesis, cell-cycle control, and DNA damage response, and repair (2, 7, 10–12). Owing to its function in cell proliferation, PCNA has been widely used as a tumor marker for cancer cell progression and patient prognosis (13–25). Given its important role in the proliferation of cancer cells, which constitute the major proliferating components in cancer-bearing patients, the inhibition of PCNA can also result in suppression of cancer progression. However, the regulation of PCNA function in cancer cells is not fully understood, making it difficult to identify the appropriate molecular niche to inhibit this abundant nuclear protein.

PCNA exists in 2 distinct forms: the replication-competent chromatin-bound form and the chromatin-unbound form which is not engaged in DNA synthesis (26). We previously reported that chromatin-bound

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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PCNA, but not the unbound form, is phosphorylated at Y211 (phospho-Y211) by the EGF receptor (27). This phosphorylation event is upregulated during S phase of the cell cycle. Further study showed that this phosphorylation enhances PCNA activity in DNA replication and DNA damage repair partly by increasing the stability of PCNA to chromatin. Thus, Y211 phosphorylation is a potential target for the specific modulation of the proliferation-active form of PCNA.

In this study, we show that phosphorylation of Y211 is a frequent event observed in human prostate cancer. Moreover, we found that Y211 phosphorylation was inhibited by *in vitro* and *in vivo* treatment with a permeable peptide specific to the Y211 motif. Downregulation of Y211 phosphorylation in prostate cancer cells resulted in inhibition of cell growth and tumor development in a xenograft model. These results provide a proof of concept for the idea that targeting Y211 phosphorylation of PCNA can be an efficient approach to prostate cancer treatment.

Materials and Methods

Cell culture, peptides, and antibodies

The cell lines PC3, DU145, and LNCaP were purchased from American Type Culture Collection and have been characterized recently (<6 months) using short tandem repeat profiling (Johns Hopkins University Fragment Analysis Facility, Baltimore, MD). All cells were maintained in DMEM/F12 (1:1) with 10% FBS. The Y211F (Ac-CGRKKRRQRRRGTFALRFLNFFTK-$CONH_2$) and Scramble (Ac-CGRKKRRQRRRGFLFTNKLFRTAF-$CONH_2$) peptides were synthesized at the Keck Peptide-synthesis Facility of Yale University, New Haven, CT. The following antibodies used in this study were purchased: α-tubulin (Sigma); cyclin B (BD Pharmingen), cyclin E and PCNA (Santa Cruz), phospho-Y211 PCNA (Bethyl).

Immunohistochemical staining

Prostate cancer tissue microarray (Imgenex; Catalog number IMH-303) was dewaxed by baking at 62°C for 1 hour. Antigen retrieval was done by heating with a microwave in 10 mmol/L of citrate (pH 6.0). The slides were then incubated with an anti-phospho-Y211 PCNA antibody overnight at room temperature, followed by incubation with biotin-conjugated secondary antibody. The immunocomplexes were then stained with avidin–biotin–peroxidase complex and amino-ethyl carbazole chromogen. Samples with no primary antibody or an IgG control antibody served as negative controls. The mean intensity of phospho-Y211 staining in each tissue section was obtained by multiplying the relative intensity score (0–3) by the percentage of epithelial cells staining positive for phospho-Y211.

Cell viability and proliferation analysis

A CellTiter-Glo luminescent cell viability assay kit (Promega) was used following the manufacturer's instruction to assess the effects of the treatments on cell viability. For each experiment, 1,000 to 3,000 cells per well were plated in 96-well plates in triplicate. The experiments were repeated at least 3 times. Cell proliferation was assessed using a colorimetric BrdU proliferation kit by following the manufacturer's instructions (Roche; Catalog no. 11647229001). Briefly, cells treated with the peptides were labeled with BrdU for 3 to 4 hours. The genomic DNA was then fixed and denatured, then incubated with the peroxidase-conjugated anti-BrdU antibody for 90 minutes. The substrate of the conjugated peroxidase was then added and the reaction product was measured by the absorbance ($A_{370\,nm} - A_{492\,nm}$). The results were then normalized by the number of total viable cells, which was determined by a side-by-side cell viability assay as described above.

Cell-cycle and annexin V staining analyses by flow cytometry

Cells were harvested by trypsin, washed with PBS, and then fixed in 70% ethanol. The fixed cells were stained with 25 μg/mL of propidium iodide (Sigma) in the presence of 1 μg/mL RNase (Sigma). For fluorescence-activated cell sorting (FACS) analysis, data were collected using a FACSCalibur flow cytometer and analyzed by the software ModFit (Verity). The cell-cycle distribution was evaluated by counting greater than 10,000 cells per sample. For annexin V staining, cells treated with the peptides were stained with APC (adenomatous polyposis coli)-conjugated annexin V for 15 minutes in the dark before processing to flow cytometry following the manufacturer's instructions (BD Biosciences).

Immunoprecipitation and Western blotting analysis

Cells were lysed by incubation with the NETN buffer [150 mmol/L of NaCl, 1 mmol/L of EDTA, pH 8.0, 20 mmol/L of Tris, pH 8.0, 0.5% NP-40, 25 mmol/L of NaF, 2 mmol/L of Na_3VO_4, 20 μL/mL aprotinin (Sigma), and 0.1 mol/L of PMSF]. For Western analysis, the lysates were separated in acrylamide gels, transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad), and probed with the indicated antibodies. Bands were visualized by a chemiluminescence-based detection method (Fisher/Pierce) that used horseradish peroxidase-conjugated secondary antibodies. For immunoprecipitation, 1 to 2 mg of protein was used for each reaction. Proteins were incubated with the antibody at 4°C overnight. Protein G agarose was then added to precipitate the antibody protein complex. The beads were then washed 4 times with NETN buffer. The immunocomplexes were then released by boiling in 2× loading buffer followed by Western blotting analysis, as described. Band intensities were quantitated by using NIH Image software.

In vivo tumor growth

PC3 cells (2.5×10^6 in 100 μL of sterile Dulbecco's phosphate-buffered saline) were inoculated into nu/nu mice by subcutaneous injection into the flanks. Each

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group included 4 mice inoculated with tumor cells on both flanks ($n = 8$). When the tumors were palpable, mice were grouped randomly into 3 groups with 4 mice in each group. The mice were then treated with control vehicle, the control scramble peptide, or the Y211F peptide, by intratumoral injection. Tumor growth was monitored weekly by measuring tumor perpendicular diameters. Tumor volume (V) was calculated using the following formula: $V = \text{length} \times \text{diameter}^2 \times 0.5$.

Statistical analysis

Data from each assay were expressed as means ± SD ($n = 3$). Statistical differences between 2 groups were determined by the Student's t test. $P < 0.05$ was considered significantly different.

Results

To assess the extent of PCNA Y211 phosphorylation in prostate cancer, a tissue microarray (TMA), consisting of tumor tissue derived from human prostate cancer patients, was screened by immunohistochemical (IHC) staining using the anti-phospho-Y211 antibody. In this array, 18 of 38 (47.4%) tumors scored high for Y211 phosphorylation whereas 20 of 38 (52.6%) scored with negative to low for Y211 phosphorylation (Fig. 1 and Table 1). Although the small sample size prohibited the evaluation of whether there was a statistically significant correlation between phospho-Y211 PCNA staining and pathologic parameters, we did note that all the stage IV patients ($n = 3$) scored high for Y211 phosphorylation.

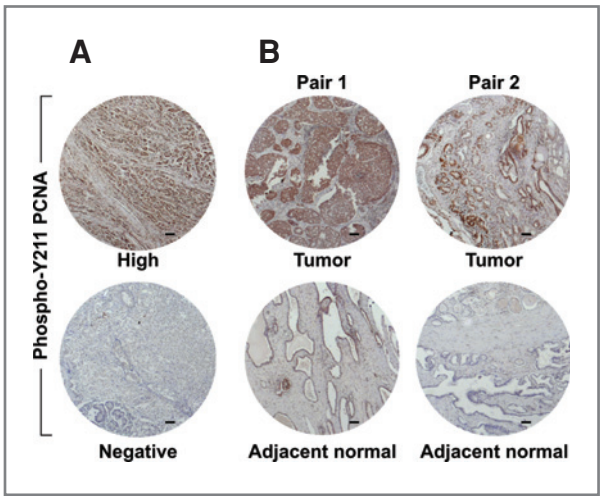

Figure 1. Phosphorylation of PCNA at Y211 is frequently observed in prostate cancer. Human prostate cancer tissues were stained with an anti-phospho-Y211 PCNA antibody. A, representative micrographs of stained specimens are shown. Top, a tissue section that was scored high in phospho-Y211 PCNA; bottom, a tissue section negative for phospho-Y211 PCNA. Bar = 0.1 mm. B, two tumor-adjacent normal tissue pairs. Pair 1, the tumor (top) and adjacent normal tissue (bottom) are both positive for phospho-Y211 PCNA staining; Pair 2, the tumor tissue is positive for phospho-Y211 PCNA staining (top) but the adjacent normal tissue is negative (bottom). Bar = 0.1 mm.

Table 1. Phospho Y211 PCNA expression in human prostate specimens

PY211 expression	% samples	Mean intensity	Intensity range
High	47.4 (18/38)	202.5	120–285
Undetectable to low	52.6 (20/38)	40.75	0–80

NOTE: The percentage of human prostate specimens that stained positive for phospho-Y211 expression and the mean intensity of phospho-Y211 staining are shown. The number of phospho-Y211–expressing samples over the total number of samples examined is shown in parentheses.

Importantly, this study also showed that Y211 phosphorylation of PCNA is a frequent event in prostate cancer. Interestingly, positive phospho-Y211 staining was observed in the 6 of 7 matched adjacent normal tissues included in the TMA (examples of positive and negative staining are shown in Fig. 1). In total, our IHC study suggests that phosphorylation of PCNA at Y211 is found in about half of human prostate cancers.

We previously showed that Y211 phosphorylation can be specifically inhibited by using a synthetic peptide with the same 12 amino acid sequence derived from the proximal region of the Y211 residue of PCNA, except with the tyrosine (Y) residue replaced by a phenylalanine (F; ref. 27). The sequence was fused with the 10 amino acid TAT peptide for efficient cell transduction and nuclear entry (28, 29). To examine the function of PCNA Y211 phosphorylation in prostate cancer cells, we treated a panel of prostate cancer cells with the chimeric TAT-Y211F peptide (referred to hereafter as the Y211F peptide). As a negative control, we used the TAT peptide fused with the same amino acid residues as in the 12 amino acid Y211F sequence, but in scrambled order (referred to hereafter as the scramble peptide). Treatment with the Y211F peptide, but not with the same dose of the control scramble peptide, resulted in inhibition of Y211 phosphorylation of PCNA in the prostate cancer cells PC3, LNCaP, and DU145 (Fig. 2). Y211 phosphorylation is

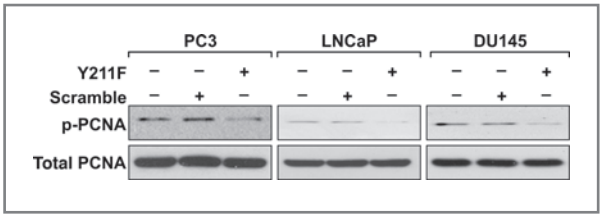

Figure 2. The Y211F peptide inhibited Y211 phosphorylation of PCNA in prostate cancer cells. The prostate cancer cell lines PC3, LNCaP, and DU145 were treated with 15 μmol/L of the Y211F peptide or the scramble peptide or vehicle alone as controls for 12 hours. Cells were then lysed and phospho-Y211 PCNA was immunoprecipitated by using the anti-phospho-Y211 antibody and was examined by Western blotting with the anti-PCNA antibody. The input amount of PCNA in the lysates was also determined.

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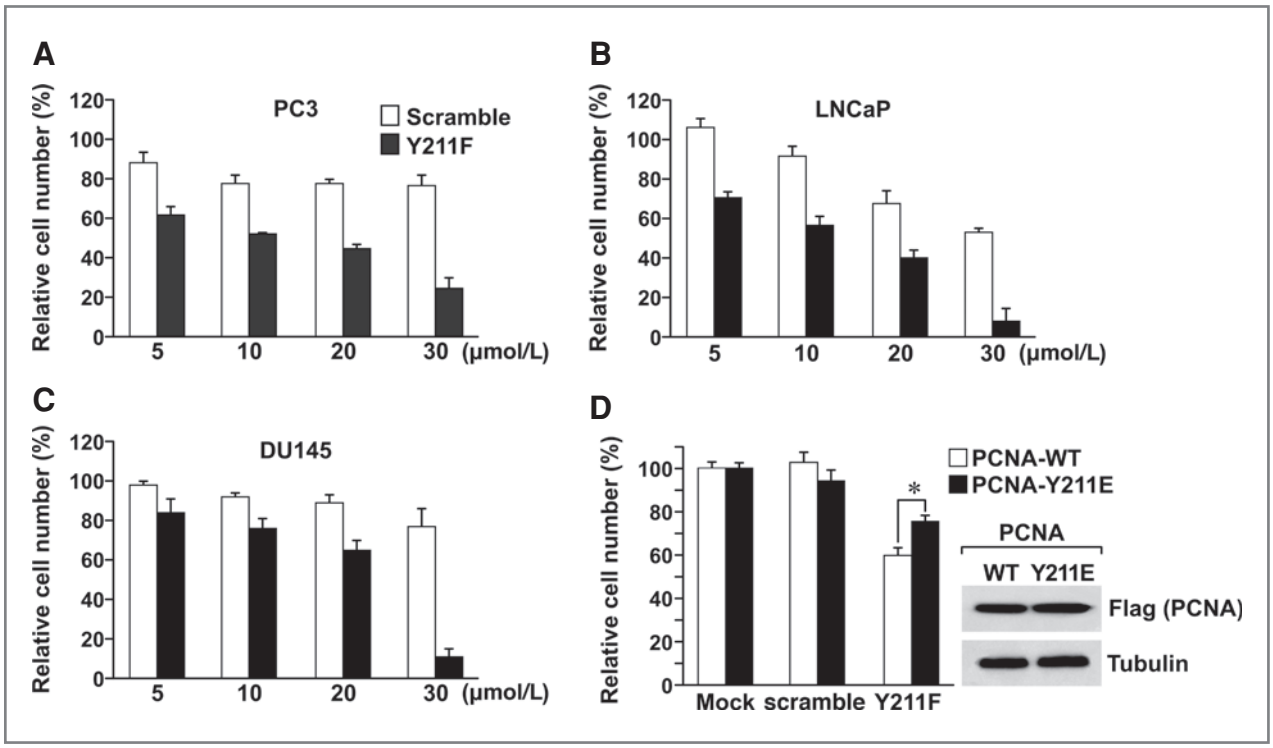

Figure 3. Inhibition of Y211 phosphorylation suppressed prostate cancer growth. The prostate cancer cell lines PC3 (A), DU145 (B), and LNCaP (C) were plated in 96-well plates and then incubated for 48 hours with the indicated peptides at different concentrations. The numbers of viable cells exposed to each treatment was then determined and expressed relative to vehicle-treated cells. D, phosphomimetic PCNA was resistant to growth inhibition mediated by the Y211F peptide. PC3 cells were transiently transfected with FLAG-tagged wild-type (WT; black bars) or Y211E (white bars) PCNA, and then treated with the Y211F peptide or the scramble control peptide at 5 μmol/L or vehicle alone for 10 hours. Left, cells expressing the Y211E phosphomimetic mutant PCNA were less sensitive to the growth inhibition effect of the Y211F peptide than cells expressing the wild-type PCNA, as determined by the cell viability assay. *, $P < 0.05$. Right, expression levels of the transfected PCNA were determined by Western blot analysis.

known as a hallmark of proliferating cells in the S phase of the cell cycle (27). Consistent with the important function of Y211 phosphorylation of PCNA in cell growth, treatment with the Y211F peptide, but not the control scramble peptide or mock treatment, significantly inhibited growth of these prostate cancer cells in a dose-dependent manner (Fig. 3). To determine whether the Y211F peptide blocks cell growth through inhibition of PCNA Y211 phosphorylation, we tested whether the phosphomimetic mutant PCNA/Y211E could rescue the growth inhibition induced by the Y211F peptide. We reasoned that, if the Y211F peptide inhibited cell growth by inhibiting Y211 phosphorylation, cells expressing the Y211E mutant PCNA should have increased resistance to inhibition by the Y211F peptide. Indeed, PC3 cells expressing the PCNA/Y211E mutant were partially resistant to the growth inhibition effects of the Y211F peptide in comparison with cells expressing the wild-type PCNA (Fig. 3D). This result supports the idea that the effect of the Y211F peptide is specific to the phosphorylation event at Y211 of PCNA.

To further analyze the mechanism of cell growth inhibition by the Y211F peptide, prostate cancer cells treated with the peptides were subjected to flow cytometry analysis (Fig. 4). Treatment with the Y211F peptide, but not with the control scramble peptide or vehicle alone (Mock), had a prominent impact on cell-cycle progression, with an accumulation of cells in the S phase of the cell cycle in PC3 cells (Fig. 4A and Supplementary Table S1). However, the Y211F peptide-treated cells were not actively engaged in proliferation, as determined by BrdU incorporation analysis, suggesting that these cells were arrested in the S phase (Fig. 4B). Consistent with this observation, expression of cyclin E, the primary cyclin governing S-phase progression, was decreased in the Y211F peptide-treated cells, whereas expression of cyclin B, which controls the progression of the M phase of the cell cycle, was not altered by the treatment (Fig. 4C). In addition to the cytostatic effect, treatment with the Y211F peptide resulted in a significant increase in the sub-G_0 fraction, an indication of enhanced apoptosis (Fig. 4D). This is consistent with an increase in the annexin V-positive cell population in response to treatment with the Y211F peptide, but not to mock treatment or treatment with the scramble control peptide (Fig. 4E). Similar effects were observed in LNCaP (Supplementary Figs. S1 and S2) and DU145 cells (Supplementary Figs. S3 and S4). In each case, as observed in PC3 cells, treatment

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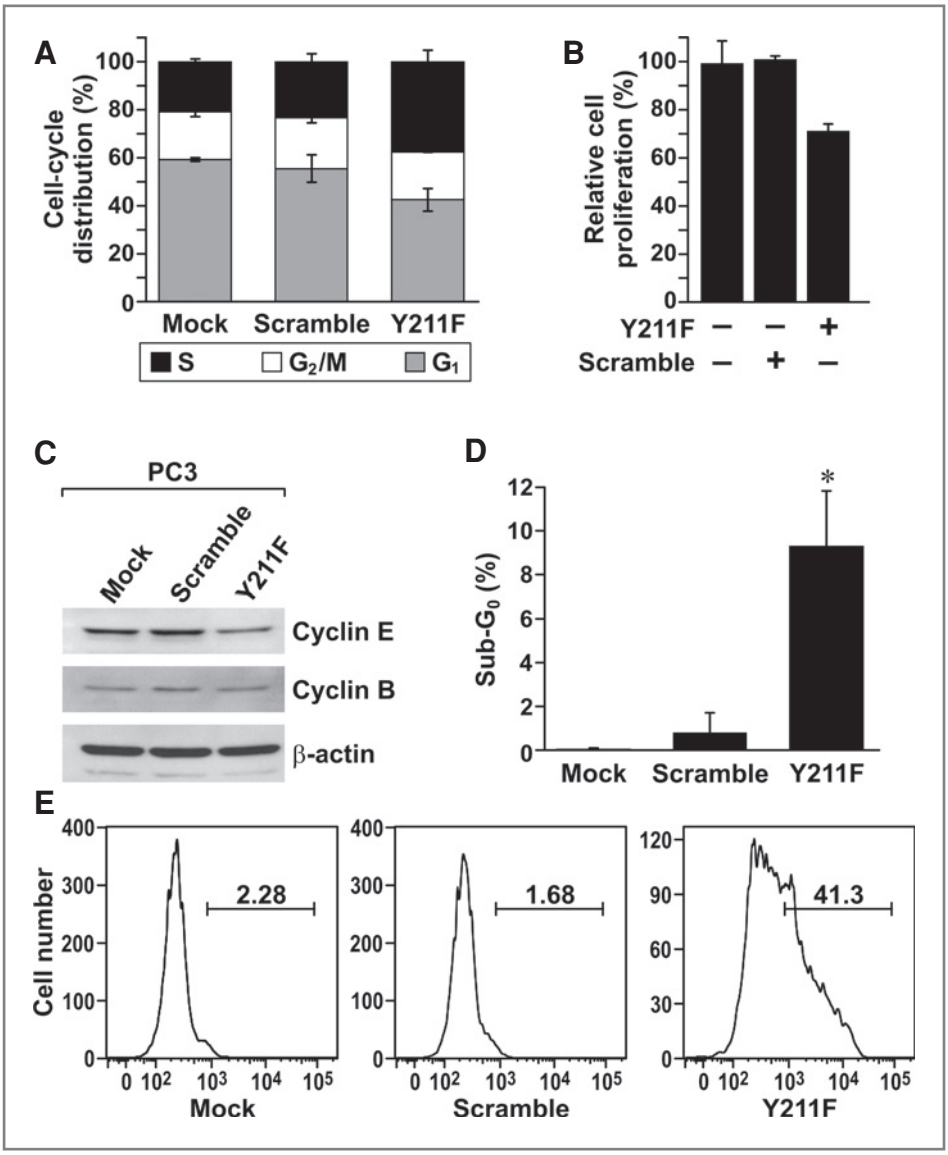

Figure 4. Y211F peptide treatment induced S-phase arrest, reduced cell proliferation, and induced cell death. A, PC3 cells mock treated with vehicle alone, or treated with the scramble or Y211F peptide (15 μmol/L) were subjected to flow cytometry analysis. The percentage of cells in the G_1, S, and G_2/M phases were plotted. B, DNA synthesis activity in the treated cells was determined by a colorimetric BrdU-incorporation analysis. For each data point, the amount of cells was normalized by a side-by-side assay for viable cells. C, expression of cyclin E and cyclin D was determined by Western analysis. β-Actin was measured as the internal control. D, the graph shows the proportion of PC3 cells in the sub-G_0 phase, as assessed by flow cytometry, after the indicated treatment. *, $P < 0.05$. E, to confirm cell death, cells were labeled with annexin V after the indicated treatment, and the numbers of positive cells were measured by flow cytometry.

with the Y211F peptide, but not the scramble peptide or mock treatment, resulted in an accumulation of cells in the S phase, accompanied by decreased cell proliferation as indicated by reduced BrdU incorporation.

Given the important role of Y211 phosphorylation in the growth function of PCNA, we asked whether the inhibition of cell growth induced by the Y211F peptide in cultured cancer cells could be recapitulated *in vivo*. To do this, we tested the growth-suppressing activity of the peptide in tumor xenografts derived from PC3 cells implanted subcutaneously into nude mice (Fig. 5). Treatment by the Y211F peptide, but not by the mock treatment with the vehicle alone or by the control scramble peptide, significantly suppressed tumor growth, as measured by tumor volume (Fig. 5A), as well as by the actual weight of isolated tumors (Fig. 5B and C). IHC staining using the anti-phospho-Y211 antibody confirmed the downregulation of Y211 phosphorylation of PCNA in the tumors injected with the Y211F peptide, in contrast to the tumor tissues injected with vehicle alone or the scramble peptide (Fig. 5D). These results corroborate with the *in vitro* data and show the importance of Y211 phosphorylation in prostate cancer cell growth as well as its potential as a target for cancer therapy.

Discussion

Enhanced proliferation provides the essential growth advantage to cancer cells of the primary tumor and at metastatic lesions. This high proliferative potential has

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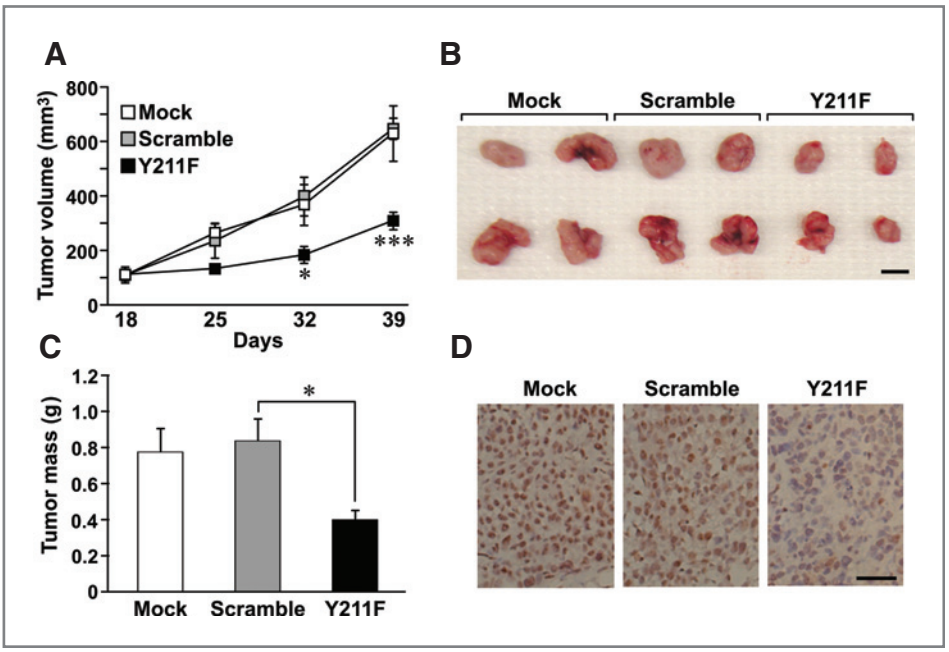

Figure 5. Y211 peptide treatment suppressed tumor growth and Y211 phosphorylation in a xenograft model of prostate cancer tumors. A, 2.5 × 106 PC3 cells were injected subcutaneously into the flanks of nude mice. When the tumors were palpable, mice were grouped randomly into 3 groups with 4 mice in each group. The mice were then treated with control vehicle (Mock), the control peptide (Scramble), or the Y211F peptide (Y211F) by intratumoral injection (3 times per week). Tumor growth was monitored weekly by measuring tumor size. *, $P < 0.05$; ***, $P < 0.001$. B, tumors were isolated from the mice at the end of treatment. Photographs of representative tumors are shown. Bar = 1 cm. C, the weight of each of the harvested tumors was measured and plotted. Treatment with the Y211F peptide significantly decreased the size of the tumors. *, $P < 0.05$. D, tumor tissues were fixed by formaldehyde and embedded in paraffin. Tissues sections were then stained with the anti-phospho-Y211 PCNA antibody. Bar = 50 μm.

been exploited as the major therapeutic target by conventional chemotherapy, which is the most commonly used frontline therapeutic approach for many cancer types. However, chemotherapy is not often used in the treatment of prostate cancer. Part of the reason is that these genotoxic agents induce DNA damage in both normal cells and cancer cells and, therefore, can result in high nonspecific cytotoxicity, which can be particularly harmful to older patients. Thus, identifying and specifically targeting proliferative cancer cells is an important means to targeting metastatic hormone-refractory prostate cancer. We have shown previously that Y211 phosphorylation of PCNA is an essential signaling event that maintains the function of PCNA on the chromatin during DNA replication (27). The current study shows that targeting the DNA synthesis machinery by blocking Y211 phosphorylation of PCNA is a promising approach.

We show that Y211 phosphorylation of PCNA is a frequent event in advanced prostate cancer. In each of the 3 prostate cancer cell lines tested, inhibition of the phosphorylation by using a cell-permeable PCNA inhibitor peptide resulted in growth arrest accompanied by blockage of cell-cycle progression through the S phase, which was confirmed by BrdU incorporation assays. In addition, expression of the S phase indicator, cyclin E, was downregulated by the peptide. We further showed that the inhibition effect can be partially rescued by the phosphomimetic Y211E mutant of PCNA, suggesting that the growth inhibition effect of Y211F peptide is mediated through a PCNA-directed mechanism. This growth inhibition effect can be recapitulated in a xenograft tumor model in which tumor growth was significantly suppressed by the treatment of the Y211F peptide. Interestingly, in addition to the antiproliferation effect, downregulation of PCNA Y211 phosphorylation also resulted in cell death, which was shown by the sub-G_0 fraction of flow cytometry and the positive annexin V staining in the treated cells. It is possible that the cytotoxicity is triggered in the arrested S-phase cells by stalked replication forks, which harbor single- and double-strand breaks in the DNA. Thus, the induction of cell death may be linked to the pathway mediating the response to DNA damage. Alternatively, Y211 phosphorylation of PCNA may have a function in regulating cell viability during cell proliferation. Discriminating between these possibilities will require further understanding of the signaling pathway leading to cell death and the underlying molecular mechanism.

Expression of Y211-phosphorylated PCNA has been associated with poor overall survival in breast cancer (27). The current study suggests that Y211 phosphorylation of PCNA may be an important tumor marker in other types of malignancy. More importantly, this is

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the first study showing the potential of Y211 phosphorylation of PCNA as a promising cancer therapy target. The major challenge in prostate cancer treatment is that although the disease initially responds to antihormonal therapies, development of advanced stage with hormone-refractory tumor is a frequent and fatal outcome. Our finding that Y211 phosphorylation occurs in both hormone-dependent (LNCaP) and hormone-independent (PC3 and DU145) prostate cancer cell lines and that inhibiting Y211 phosphorylation of PCNA resulted in significant growth inhibition both *in vitro* and *in vivo* suggests that targeting Y211 phosphorylation can also be applied to the hormone-refractory prostate cancer cells. The current work is a proof of concept that targeting the Y211-phosphorylated PCNA can be an effective strategy against prostate cancer. Although the phospho-Y211–directed peptide is a rational strategy to target proliferation-competent PCNA, limitations associated with peptides as a therapeutic agent, particularly in a systemic approach, exist. Other molecular strategies, such as the conjugation of tumor-specific ligands and incorporation of structural or chemical modifications, can further improve the specificity, delivery, and stability for systemic administration. To this regard, it is noteworthy that there are numerous reported successes that provide precedent for this strategy such as the peptide inhibitor of the Jun kinase signaling pathway (30). Our results warrant further investigation to identify small molecules that can specifically target Y211 phosphorylation of PCNA. These strategies may prove to be particularly useful in targeting the proliferative potential of cells in advanced cancers that are resistant to conventional therapeutics. One concern is whether targeting PCNA, which is a ubiquitously expressed proliferation factor, could lead to severe general cytotoxicity. Our strategy targets a subpopulation of PCNA harboring a specific posttranslational modification (Y211 phosphorylation), which has been shown to be expressed in breast cancer tissues and to be significantly correlated with poor survival. In addition, cancer cells likely constitute the compartment with the highest level of cell proliferation in a cancer patient. It is therefore conceivable that targeting Y211 phosphorylation of PCNA would have a greater impact in tumor cells versus normal tissues. It should also be noted that posttranslational modification is not a requirement for PCNA to conduct DNA synthesis, as recombinant PCNA, which is believed to be devoid of such modifications, has been used for short DNA synthesis *in vitro* (31). Thus, it appears that posttranslational modifications of PCNA mainly serve as modulators of PCNA function (2). Indeed, our previous study showed that the function of phospho-Y211 is, at least in part, to enhance PCNA stability on the chromatin, therefore promoting its activity in DNA synthesis and DNA damage repair (27). Y211 phosphorylation may modulate different functions of PCNA during cell proliferation through other mechanisms. Together, targeting these functions is expected to have a major impact on cancer cells, which require a high level of proliferative activity to support rigorous growth. Furthermore, as many other oncogenic pathways also function in enhancing cancer growth and tumor progression, combination therapies that target these pathways along with phospho-Y211 of PCNA could be a new strategy for cancer therapy. Finally, the finding that phospho-Y211 PCNA is expressed in phenotypically normal tissues adjacent to tumors suggests that the signaling event can occur early in tumorigenesis. If this is the case, phospho-Y211 PCNA may signal increased growth advantage in otherwise normal tissues and could be used as a marker of early lesions. Further understanding the mechanisms regulating Y211 phosphorylation of PCNA in the tissues surrounding tumors may provide molecular insight into tumor development in prostate cancer that could be exploited to therapeutic advantage.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by any authors.

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Interaction of Proliferation Cell Nuclear Antigen (PCNA) with c-Abl in Cell Proliferation and Response to DNA Damages in Breast Cancer

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Abstract

Cell proliferation in primary and metastatic tumors is a fundamental characteristic of advanced breast cancer. Further understanding of the mechanism underlying enhanced cell growth will be important in identifying novel prognostic markers and therapeutic targets. Here we demonstrated that tyrosine phosphorylation of the proliferating cell nuclear antigen (PCNA) is a critical event in growth regulation of breast cancer cells. We found that phosphorylation of PCNA at tyrosine 211 (Y211) enhanced its association with the non-receptor tyrosine kinase c-Abl. We further demonstrated that c-Abl facilitates chromatin association of PCNA and is required for nuclear foci formation of PCNA in cells stressed by DNA damage as well as in unperturbed cells. Targeting Y211 phosphorylation of PCNA with a cell-permeable peptide inhibited the phosphorylation and reduced the PCNA-Abl interaction. These results show that PCNA signal transduction has an important impact on the growth regulation of breast cancer cells.

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Introduction

The c-Abl proto-oncogene is a multi-functional non-receptor tyrosine kinase that shuttles between the cytoplasm and the nucleus [1]. A substantial body of knowledge has been established regarding the mechanisms of c-Abl in regulating cell migration, the response to oxidative stresses, and apoptosis [2]. The c-Abl kinase was originally identified as a frequent target of oncogenic chromosomal translocation in hematopoietic neoplasia, but has been increasingly recognized for its involvement in solid tumors. In lung and breast cancers, deregulated c-Abl kinase contributes to tumor development [2–5]. In breast cancer, activated c-Abl kinase promotes cancer progression [4,6], while inhibition of c-Abl blocks the transforming phenotypes by suppressing anchorage-independent growth, inducing apoptosis, and inhibiting cell proliferation [5]. Consistent with this, our recent study showed that increased c-Abl expression is a frequent event in breast cancer (~40%) [7].

Proliferating cell nuclear antigen (PCNA) is the molecular coordinator in the core DNA synthesis machinery [8–16]. PCNA forms a homotrimeric ring encircling the DNA double helix and acts as a molecular platform to recruit proteins involved in DNA synthesis, cell-cycle control, and DNA-damage response and repair [8,13,16–18]. PCNA exists in two distinct forms: the replication-competent chromatin-bound form and the chromatin-unbound form, which is not involved in DNA synthesis [19]. Not much is known about how the two populations of PCNA are regulated. We previously reported that the chromatin-bound PCNA, but not the unbound form, was phosphorylated at Y211 (phospho-Y211) by the EGF receptor [20]. This phosphorylation event was upregulated during the S phase of the cell cycle. Further study demonstrated that this phosphorylation enhanced the stability of chromatin-associated PCNA and enhanced its activity in DNA replication.

In the current study, we identify c-Abl as a binding partner of PCNA and show that Y211 phosphorylation of PCNA serves as a binding signal for PCNA to associate with c-Abl under normal growth conditions and in cells responding to DNA-damage stresses. We further demonstrate that c-Abl promotes chromatin association of PCNA and that Y211 phosphorylation is an important cell growth-related event downstream of c-Abl.

Results

We tested whether Y211 phosphorylation of PCNA can serve as a signaling event that, in turn, regulates its function. To do this, we screened a microarray of functional domains derived from

different proteins and tested whether phospho-Y211 preferentially associated with these motifs. Synthetic peptides, encompassing the wild-type sequence surrounding the Y211 residue or the same peptide with phosphorylated Y211, were conjugated to a fluorescent dye (Cy3) and used for probing the microarray (data not shown). The Cy3-conjugated peptide with the non-phosphorylated wild-type sequence did not bind to any of the functional domains. In contrast, incubation with the phosphorylated peptide identified the SH2 domain of the non-receptor tyrosine kinase c-Abl as a phospho-Y211-binding motif. Further verification with co-immunoprecipitation (co-IP) using extracts of MDA-MB-231 and BT474 cells demonstrated that PCNA and c-Abl formed a complex in vivo (**Fig. 1**).

These results indicate that PCNA interacts with c-Abl, and suggest that the interaction is mediated by Y211 phosphorylation. To further test the importance of Y211 phosphorylation in the Abl-PCNA interaction, wild-type PCNA or mutant PCNA in which the Y211 residue was replaced with a phenylalanine (Y211F) was transfected into HEK293T cells. Expression of the transfected PCNA was at corresponding levels relative to the endogenous PCNA (**Figure S1**). Endogenous c-Abl was immunoprecipitated with a c-Abl–specific antibody and the level of co-precipitated ectopic PCNA was determined by western analysis. As shown in **Figure 2A**, binding between the Y211F mutant and c-Abl was reduced compared with that of wild-type PCNA, suggesting that phosphorylation of Y211 is important for the association between PCNA and c-Abl. To further define the role of Y211 phosphorylation of PCNA in association with c-Abl, a synthetic peptide containing the sequence of the proximal region of Y211 in which the Y211 residue was replaced with phenylalanine was used to block phosphorylation at Y211 (hereafter referred to as the Y211F peptide) [20]. As a negative control, we used a peptide with the same amino acid residues as the Y211F peptide but in a scrambled order (referred to hereafter as the scramble peptide). These peptides were conjugated with the HIV-derived TAT sequence at the N terminus, which was capable of transducing the peptides to the nuclear compartment [21]. This was demonstrated by the clear nuclear localization of 5(6)-carboxyfluorescein (FAM)-labeled Y211F peptide (comparing FAM-TAT-Y211F and FAM-Y211F in **Fig. 2B**). Importantly, treatment with the Y211F peptide, but not with the same dose of the control scramble peptide, resulted in inhibition of Y211 phosphorylation of PCNA in BT474 and MDA-MB-231 cells (**Fig. 2C**), and blocked the interaction between endogenous PCNA and c-Abl proteins in both cell lines (**Fig. 2D**). The importance of Y211 phosphorylation for c-Abl interaction was further supported by incubating cells with synthetic peptides of the wild-type sequence with and without phosphorylation at the Y211 residue (**Fig. 2E**). The results showed that the phosphorylated

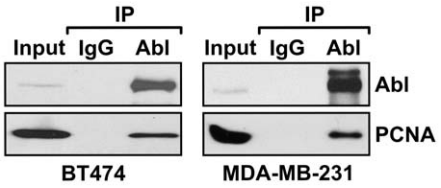

Figure 1. PCNA interacts with c-Abl in vitro and in vivo. Endogenous interaction of PCNA with c-Abl. Interaction of endogenous PCNA and c-Abl in breast cancer cell lines BT474 cells (left panel) and MDA-MB-231 cells (right panel) demonstrated by immunoprecipitation with an anti–c-Abl antibody. After gel separation, the co-precipitated PCNA and c-Abl were detected by using the corresponding antibodies.
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Y211 peptide was more potent in blocking the Abl-PCNA interaction than the non-phosphorylated counterpart. Taken together, these results indicate that phosphorylation of Y211 on PCNA is critical for the interaction between PCNA and c-Abl.

To test the impact of inhibiting Y211 phosphorylation of PCNA in breast cancer cell growth, MDA-MB-231 (**Fig. 3A**) and BT474 (**Fig. 3B**) cells were treated with the Y211F peptide or the control peptide. Treatment with the Y211F peptide inhibited growth in both cell lines in a dose-dependent manner, indicating that Y211 phosphorylation is a critical event in breast cancer cells. Indeed, targeting Y211 phosphorylation by the Y211F peptide suppressed proliferation of MDA-MB-231 (**Fig. 4**) and BT474 (**Fig. 5**) cells. Flow cytometry analysis showed that MDA-MB-231 cells treated with the Y211F peptide revealed a significantly increased proportion of cells in the S phase and decreased the proportion in the G2-M phase of the cell cycle, while cells treated with the control scramble peptide had cell cycle distributions similar to the mock-treated cells (**Fig. 4A**). The cells accumulated in the S phase were not engaged in DNA synthesis. This was revealed by BrdU incorporation analysis showing that treatment with the Y211F peptide resulted in reduced DNA synthesis activity per viable cell (**Fig. 4B**). Similar effects were observed in BT474 cells (**Fig. 5**). These results suggest that Y211 phosphorylation of PCNA conveys a growth-promoting function partly through its interaction with c-Abl. To further determine whether Y211 phosphorylation cooperates with c-Abl in enhancing cell growth, BT474 cells harboring shRNA against *c-Abl* (shAbl) or control shRNA of scrambled sequence (shCtrl) were treated with the Y211F or the scramble peptide (**Fig. 5C**). While Y211F peptide treatment significantly inhibited the growth of BT474/shLuc cells, the growth inhibition was partially rescued by knocking down c-Abl expression in BT474/shAbl cells. The reduced sensitivity of BT474/shABL cells to the Y211F peptide is consistent with the notion that c-Abl enhances cell growth at least in part through Y211 phosphorylation.

To further define the mechanism by which c-Abl–PCNA interaction is regulated and to discern the functional consequences of this interaction, cells were treated with or without ionizing irradiation. We found that the levels of Y211 phosphorylation were increased by the treatment (**Fig. 6A**). Lysates of these cells were subjected to immunoprecipitation with an anti-c-Abl antibody. The levels of co-precipitated PCNA were then determined by western analysis (**Fig. 6B**). Corroborating the observation that c-Abl interacts with PCNA through Y211 phosphorylation (**Fig. 2**), and that Y211 phosphorylation was enhanced by IR treatment (**Fig. 5A**), the interaction between c-Abl and PCNA was also enhanced by IR treatment. These results taken together suggest that Y211 phosphorylation is an important signaling event in response to DNA-damage stresses in breast cancer cells. Indeed, blocking BT474 cells with the Y211F peptide, but not the control scramble peptide, sensitized cells to IR treatment (**Fig. 6C**). Corroborating these results, further experiments demonstrated that c-Abl plays a pivotal role in promoting the chromatin association of PCNA (**Fig. 7**). MDA-MB-231/shAbl and MDA-MB-231/shCtrl cells were extracted with Triton X-100 to isolate the chromatin-bound and chromatin-unbound fractions of PCNA. Down-regulation of c-Abl resulted in a significant decrease in chromatin-bound PCNA (**Fig. 7A**). IR treatment led to a further reduction of the chromatin-bound form of PCNA in both cell lines, with the MDA-MB-231/shAbl cells containing the lowest level of chromatin-associated PCNA. Interestingly, after IR treatment, the remaining chromatin-bound PCNA responded to DNA-damage stress by forming sub-nuclear foci (**Fig. 7B**). In the absence of stress, MDA-MB-231/shAbl cells had moderately fewer PCNA foci-positive cells than the MDA-

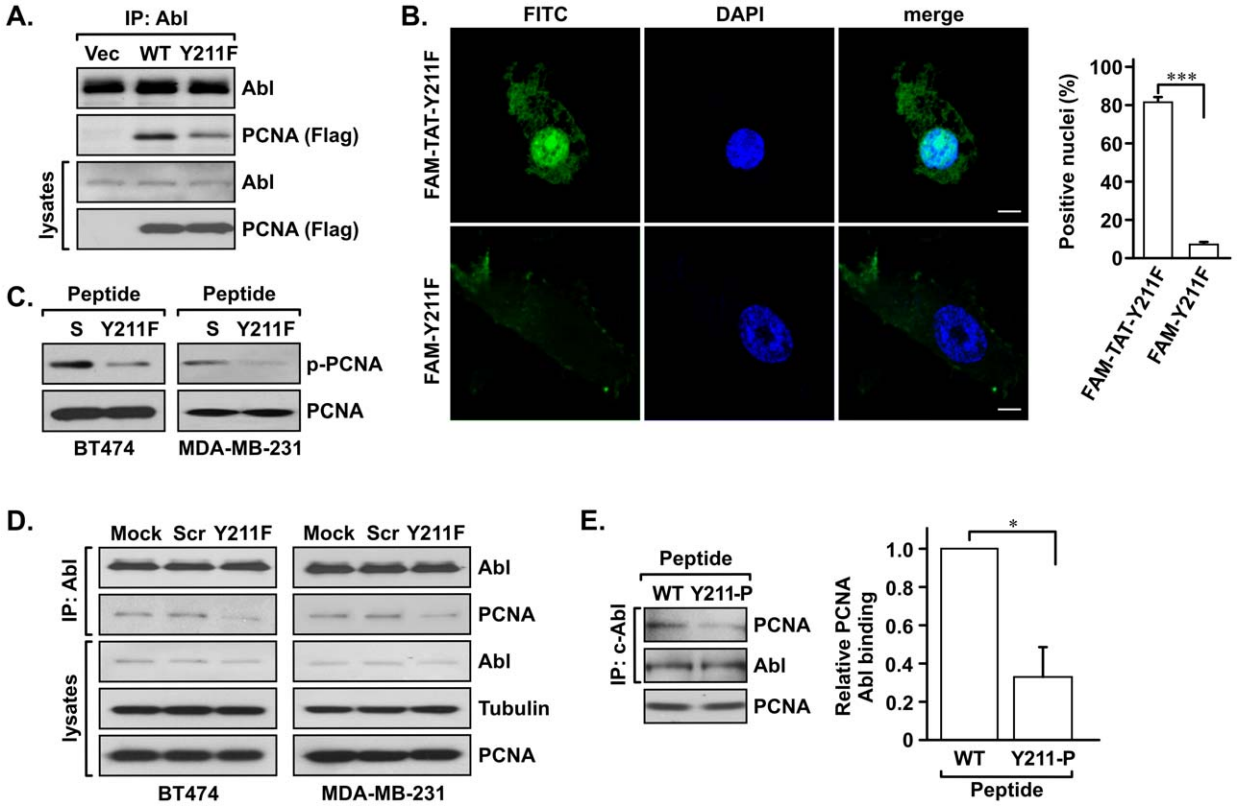

Figure 2. Y211 phosphorylation of PCNA is important for c-Abl association. A. HEK293T cells were transfected with FLAG-tagged wild-type or Y211F PCNA. Endogenous c-Abl was then immunoprecipitated with an anti-c-Abl antibody. The levels of co-immunoprecipitated FLAG-PCNA were determined by western analysis. **B.** Left panel, representative images of MDA-MB-231 cells treated for 30 min with 7.5 μM of the indicated FAM-labeled synthetic peptide with (FAM-TAT-Y211F) or without (FAM-Y211F) the TAT sequence conjugation. Localization of the peptides was determined by fluorescence confocal microscopy. Scale bar = 5 μm. Right panel, the numbers of cells with positive nucleus were counted in three independent fields for each treatment and the data are plotted. ***, $P<0.005$. **C.** The Y211F peptide inhibited Y211 phosphorylation of PCNA in breast cancer cells. The breast cancer cell lines BT474 and MDA-MB-231 were treated with 15 μM of the Y211F peptide or the control scramble peptide for 12 h. Cells were then lysed and phospho-Y211 PCNA was immunoprecipitated by using the anti-phospho–Y211 antibody, which was then detected by western blotting with an anti-PCNA antibody. The levels of input PCNA in the lysates are shown in the lower panel. **D.** The Y211F peptide inhibited the interaction between PCNA and c-Abl. Cells were treated with the peptides as described in C, and the cell lysates were then immunoprecipitated with an anti–c-Abl antibody. The co-precipitated PCNA and c-Abl were then examined by western analysis. **E.** Left panel, phospho-Y211 peptide inhibits the interaction between PCNA and c-Abl. BT474 cells were treated with the TAT-conjugated Y211 peptide with (Y211-p) or without (WT) phosphorylation at the Y211 residue. Cell lysates were immunoprecipitated with an anti-c-Abl antibody. The immune-complexes were analyzed with western blotting for the levels of interacting PCNA. Right panel, the data of three independent repeats were quantitated and plotted. *, $P<0.05$.
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MB-231/shCtrl control cells. Ionizing irradiation dramatically induced nuclear PCNA foci in MDA-MB-231/shCtrl cells. Such induction was not observed in MDA-MD-231/shAbl cells (**Fig. 7C**). Thus, depletion of c-Abl mitigates the PCNA-mediated DNA-damage response.

Discussion

Y211 phosphorylation of PCNA represents a mechanism by which growth signaling regulates nuclear proliferation events [8,20]. The post-translational modification was known to enhance PCNA stability on the chromatin. However, it was not known whether the phosphorylation event could also mediate PCNA regulatory signaling. In the current study, we demonstrated that phospho-Y211 PCNA forms a docking site for the non-receptor tyrosine kinase c-Abl and the resulting association with c-Abl enhances the level of chromatin-bound PCNA, in unperturbed as well as DNA damage-stressed cells. These results unveil the crosstalk between the DNA replication machinery and other nuclear signaling molecules to regulate cell growth. Given the versatile functions of PCNA in maintaining the integrity of the genome, further exploration of the underlying mechanisms of these functions can lead to a better understanding of how cell signaling in the nucleus shapes the dynamics of genomic stability in cancer cells.

Recently, He *et al.* reported that c-Abl interacted with PCNA through a putative PCNA-binding motif in the SH2 domain of c-Abl [22]. This proposed motif, with the amino acid sequence QRSI, is located at amino acid residues 160 to 163 of the c-Abl protein (NCBI reference sequence NP_005148.2). It was suggested by the authors that the QXXI signature is a PCNA-interacting motif. However, we tested mutant c-Abl in which that motif was mutated to ARSA, as used in that report, and found that the mutant appeared to have binding ability similar to that of the wild-type c-Abl (**Figure S2**), indicating that the PCNA did not bind to c-Abl through the QXXI motif in our experimental condition. This discrepancy could be due to different experimental conditions and/or the setting of the experiments. In the current study, the binding between the transfected c-Abl and the endogenous PCNA was assessed, while in the study by He *et al.* both proteins were ectopically expressed. It is noteworthy that although different

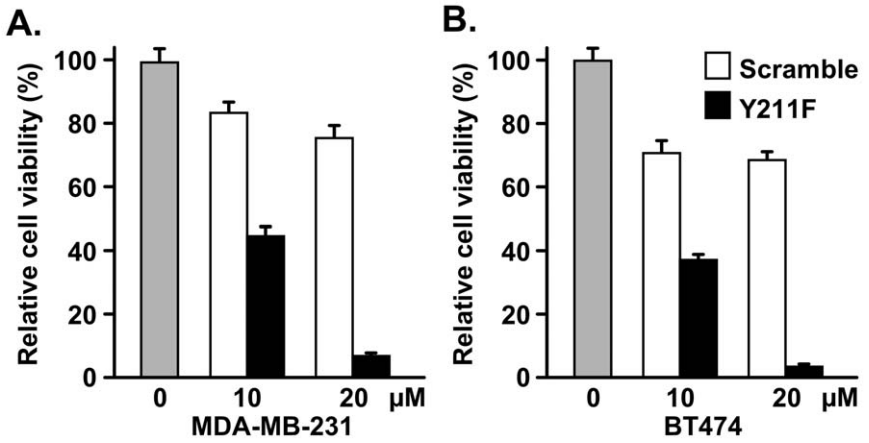

Figure 3. Y211 phosphorylation is an important growth signal downstream of c-Abl. The breast cancer cell lines MDA-MB-231 (A) and BT474 (B) were treated with the Y211F peptide or the control scramble peptide at the indicated doses for 48 hours. Surviving cells were then quantified by MTT assay and the results were plotted.
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binding sites are identified in these two studies, these findings are not necessarily mutually exclusive. For example, while the identified PCNA-binding motif of c-Abl may play a role in anti-apoptosis, interaction between Abl/SH2 with PCNA/phospho-Y211 can confer a signaling for growth advantage in cell proliferation. Further experiments using the corresponding c-Abl and PCNA mutants under unperturbed and stressed conditions should be able to test and characterize these two interaction modes.

Our previous study showed that expression of Y211-phosphorylated PCNA was associated with poor overall survival in breast cancer [20]. We recently reported that c-Abl expression is a frequent event in breast cancer [7]. The current study provides insight into the crosstalk between these two breast cancer markers. We reasoned that if phospho-Y211 PCNA has a major contribution to the growth-promoting function of c-Abl, down-regulation of Y211 phosphorylation should have a more significant impact on cells expressing c-Abl than cells lacking the c-Abl function. Indeed, Y211F peptide treatment conferred a more significant growth inhibition effect in cells expressing c-Abl than in the cells in which c-Abl was depleted (**Fig. 5C**). Such functional interaction between c-Abl and PCNA is also supported by the observation in **Figure 7** in which c-Abl depletion resulted in mitigated foci formation of PCNA in response to DNA damage. These results warrant further study to determine the role of Y211 phosphorylation of PCNA in response to growth stimulation and DNA-damaging stresses. Given that enhanced proliferation provides an essential growth advantage to cancer cells in primary tumors and in metastatic lesions and that cancer cells likely constitute the major proliferative compartment in a cancer patient, exploiting the proliferative function of PCNA for therapeutic gains has high potential to be effective in cancer treatment. We recently reported that phosphorylation of PCNA at Y211 is a promising treatment target in prostate cancer [23]. Our current results suggest that targeting phospho-Y211 PCNA could be an effective strategy in breast cancer treatment as well.

Materials and Methods

Cell culture, peptides, and antibodies

The breast cancer cell lines MDA-MB-231 and BT474 were purchased from American Type Culture Collection (ATCC) and grown in DMEM/F12 (1:1) with 10% fetal bovine serum. The

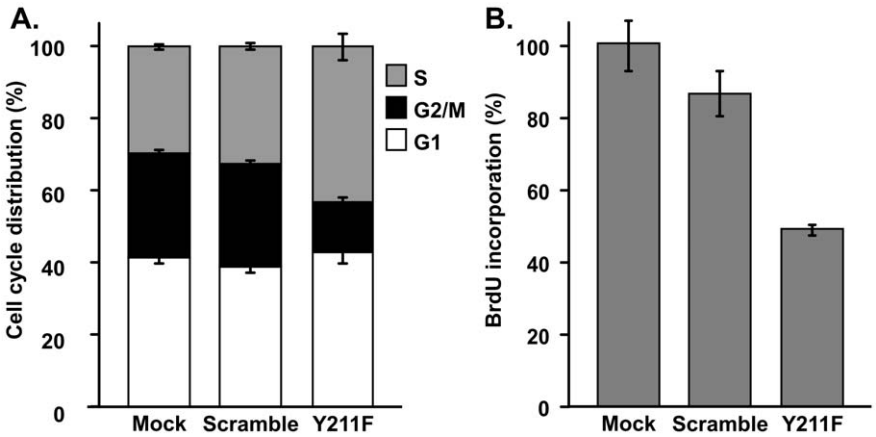

Figure 4. Targeting breast cancer cell line MDA-MB-231 with the Y211F peptide resulted in growth inhibition. A. Cells mock-treated with vehicle alone or treated with the scramble or Y211F peptide (15 μM) were subjected to flow cytometry analysis. The percentages of cells in the G1, S, and G2/M phases were plotted. **B.** DNA synthesis activity in the treated cells was determined by a colorimetric BrdU-incorporation analysis. For each data point, the amount of incorporated BrdU was normalized to the percentage of viable cells, as determined by a side-by-side MTT assay.
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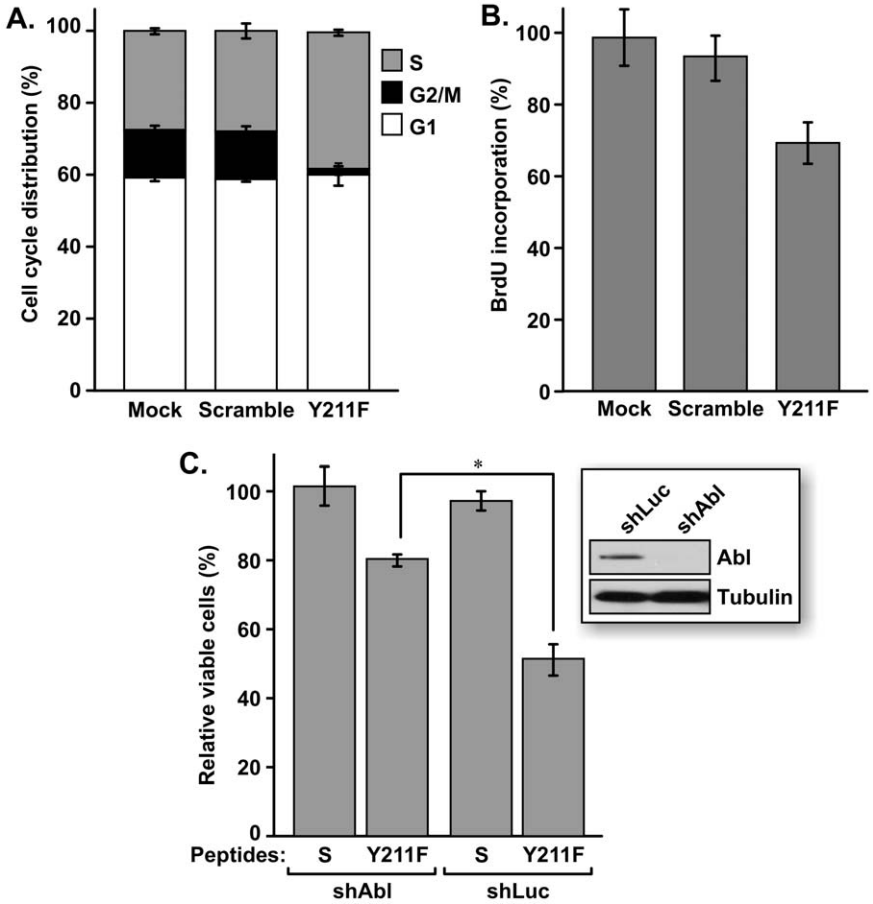

Figure 5. Growth inhibition of BT474 cells by targeting Y211 phosphorylation of PCNA. A. Cells mock-treated with vehicle alone or treated with the scramble or Y211F peptides (15 μM) were subjected to flow cytometry analysis. The percentages of cells in the G1, S, and G2/M phases were plotted. **B.** DNA synthesis activity in the treated cells was determined by a colorimetric BrdU-incorporation analysis. For each data point, the amount of incorporated BrdU was normalized to the percentage of viable cells, as determined by a side-by-side MTT assay. **C.** Depletion of c-Abl decreases sensitivity to Y211F peptide-mediated growth inhibition. A derivative of BT474 cells harboring an shRNA against c-Abl (BT474/shAbl) or luciferase (BT474/shLuc) were treated with 10 μM Y211F peptide or the scramble peptide for 48 h. Surviving cells were then assessed by MTT assay and the results were plotted. *, $P<0.05$.
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following peptides were synthesized at the Keck Peptide-synthesis Facility of Yale University: TAT-conjugated peptides including the wild-type Y211 non-phosphorylated peptide (WT; Figure 2E) (Ac-CGRKKRRQRRRGTFALRYLNFFTK-$CONH_2$), the wild-type Y211-phosphorylated peptide (Y211-P; Figure 2E) (Ac-CGRKK-RRQRRRGTFALRYpLNFFTK-$CONH_2$), the Y211F peptide (Ac-CGRKKRRQRRRGTFALRFLNFFTK-$CONH_2$), the scrambled control peptide (Ac-CGRKKRRQRRRGFLFTNKLFRTAF-$CONH_2$), the purified N-terminally FAM-conjugated TAT-Y211F peptide (FAM-TAT-Y211F; Figure 2B), and the Y211F peptide without TAT sequence (FAM-Y211F; Figure 2B) were. The following antibodies used in this study were purchased from a variety of manufacturers: α-tubulin (Sigma; St. Louis, MO); c-Abl (BD Pharmingen; San Jose, CA), Histone H3, PCNA (Santa Cruz; Santa Cruz, CA), phospho-Y211 PCNA (Bethyl; Montgomery, TX).

Proliferation analysis

Cell proliferation was assessed by using a colorimetric BrdU proliferation kit according to the manufacturer's instructions (Roche, Indianapolis, IN; Cat. No. 11647229001). Briefly, 1000 to 3000 cells per well were plated in 96-well plates in triplicate. The experiments were repeated at least three times. Cells treated with the peptides were labeled with BrdU for 3 to 4 h. The genomic DNA was fixed and denatured, and then incubated with peroxidase-conjugated anti-BrdU antibody for 90 min. A substrate for the conjugated peroxidase was then added and the reaction product was quantified by measuring the absorbance. The results were then normalized by the number of total viable cells, which was determined by a side-by-side cell-viability assay, as described above.

Cell-cycle analyses by flow cytometry

Cells were harvested by trypsin, washed with PBS, and then fixed in 70% ethanol. The fixed cells were stained with 25 μg/ml propidium iodide (Sigma; St. Louis, MO) in the presence of 1 μg/ml RNase (Sigma; St. Louis, MO). For fluorescence-activated cell sorting (FACS) analysis, data were collected using a FACSCalibur flow cytometer and analyzed using the software ModFit (Verity; Topsham, ME). The cell-cycle distribution was evaluated by counting >10,000 cells per sample.

Immunoprecipitation and western blotting analysis

Cells were lysed by incubation with NETN buffer (150 mM NaCl, 1 mM EDTA pH 8.0, 20 mM Tris pH 8.0, 0.5% NP-40,

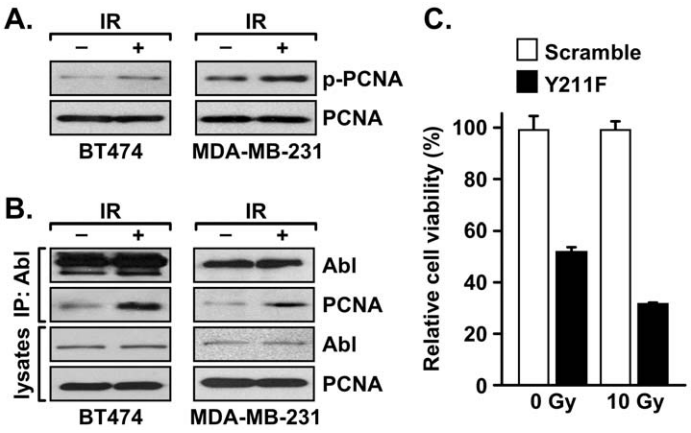

Figure 6. IR induced Y211 phosphorylation of PCNA and its interaction with c-Abl. A. BT474 and MAD-MB-231 cells were irradiated (10 Gy), and then incubated for 2 h. Cell lysates were immunoprecipitated by using the anti-phospho-Y211 antibody and then probed by western blotting with an anti-PCNA antibody. The input amount of PCNA in the lysates was also assessed (lower panel). **B.** IR induced the interaction between PCNA and c-Abl in breast cancer cells. Cells were treated as described in A, and then lysed. Endogenous c-Abl was immunoprecipitated by using an anti–c-Abl antibody, and the co-precipitated PCNA and c-Abl were detected by western analysis. **C.** BT474 cells were treated with the Y211F peptide or the scramble peptide (10 μM) for 12 h, then irradiated (10 Gy), followed by incubation for 36 h. Surviving cells were then assessed by MTT assay. Relative cell viability (*y* axis) denotes reduced cell survival compared with the scramble control, which is defined as 100%.
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25 mM NaF, 2 mM Na_3VO_4, 20 μl/ml aprotinin (Sigma), 0.1 M PMSF). For western analysis, the lysates were separated in acrylamide gels, transferred to a PVDF membrane (Bio-Rad; Hercules, CA), and probed with the indicated antibodies. Bands were visualized by a chemiluminescence-based detection method (Fisher/Pierce; Rockford, IL) that used horseradish peroxidase-conjugated secondary antibodies. For immunoprecipitation, 1 to 2 mg of protein was used for each reaction. Proteins were incubated with the antibody at 4°C overnight. Protein G agarose was then added to precipitate the antibody-protein complex. The

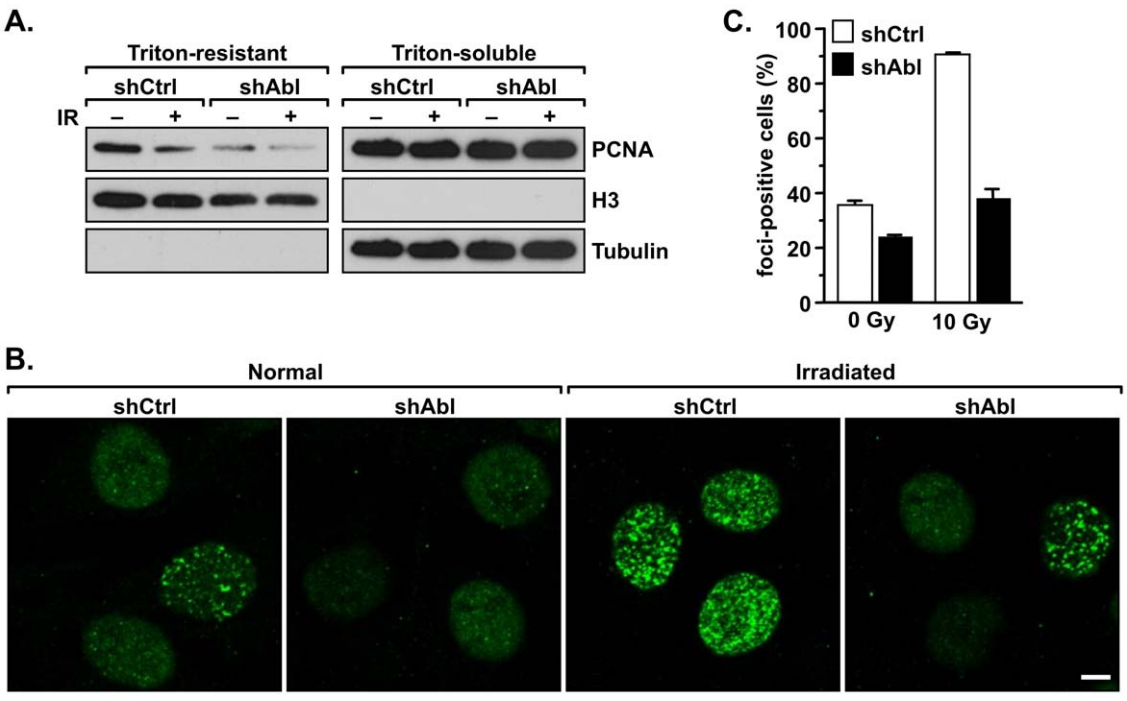

Figure 7. c-Abl enhances chromatin association and PCNA foci formation in response to DNA damage. A. MDA-MB-231/shAbl and MDA-MB-231/shCtrl cells were exposed to 10 Gy of IR. Following one hour of incubation, the cells were extracted with 0.5% of Triton X-100. Levels of PCNA in the soluble and insoluble fractions were examined by western analysis. Expression of α-tubulin and histone H3 was assessed as markers of the soluble and insoluble fractions, respectively. **B.** c-Abl is important in the formation of PCNA nuclear foci. MDA-MB-231/shAbl and MDA-MB-231/shCtrl cells were mock-treated or irradiated with 10 Gy of IR followed by incubation for one hour. Cells were then fixed with methanol and stained with an anti-PCNA antibody. **C.** To evaluate the number of foci-positive cells, five independent fields representing each treatment were counted. The experiment was repeated two times, and the results of the two trials were consistent.
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beads were then washed four times with NETN buffer. The immunocomplexes were then released by boiling in 2× loading buffer followed by western blotting analysis, as described.

Immunofluorescence confocal microscopy

Cells were fixed and then permeabilized by methanol and 0.5% Triton X-100 for 10 minutes at room temperature. After four washes with PBS, the cells were blocked with 10% normal goat serum for 1 hour at room temperature, and then immunostained with primary antibody against PCNA (1:200 dilution in PBS with 0.2% BSA) overnight at 4°C. After three washes with PBS, the FITC-conjugated secondary antibody was applied for 45 minutes at room temperature. Images were captured with a Zeiss laser scanning confocal microscope (LSM510). For localization analysis of the FAM-labeled peptides, cells were treated with the peptide at 7.5 μM for 30 min. Treated cells were then washed twice with PBS, and fixed with 4% paraformaldehyde. Peptide localization was visualized by fluorescence confocal microscopy as described above.

Statistical analysis

Data from each assay were expressed as means ± SD (n = 3). Statistical differences between two groups were determined by the Student's t-test. $P<0.05$ was considered significantly different.

Supporting Information

Figure S1 Expression of FLAG-PCNA in HEK293T cells. Cells were transfected with FLAG-PCNA (wild-type, Y211F) or the empty vector (pcDNA3). The lysates of the transfected cells were analyzed by western blotting using an anti-PCNA antibody (Santa Cruz; Santa Cruz, CA). Both the endogenous (endo PCNA) and the FLAG-tagged ectopic PCNA (Flag-PCNA) were shown as indicated by the arrows.
(TIF)

Figure S2 Assess the putative PCNA-binding motif of c-Abl. The c-Abl mutant c-Abl/QI-AA had no effect on the interaction between PCNA and c-Abl. HEK293T cells were transfected with the cDNA of the Myc-tagged wild-type or QI-AA mutant of c-Abl or the control vector. Cell lysates were then immunoprecipitated with an anti-Myc antibody. The levels of co-precipitated endogenous PCNA were determined by western analysis using an anti-PCNA antibody.
(TIF)

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Author Contributions

Conceived and designed the experiments: S-CW. Performed the experiments: HZ Y-HL P-CH AE. Analyzed the data: HZ MB M-CH S-CW. Contributed reagents/materials/analysis tools: MB M-CH S-CW. Wrote the paper: S-CW.

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